

UNIVERSITY OF ALBERTA

**MATRIX METALLOPROTEINASE ACTIVATION DOWNSTREAM OF tPA
THROMBOLYSIS AND THE EFFECTS ON THE BLOOD-BRAIN BARRIER**

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of *Doctor of Philosophy*

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Abstract

Thrombolysis with tissue plasminogen activator (tPA) is the only Health Canada approved pharmacological therapy for the treatment of acute ischemic stroke. Administration of tPA to stroke patients is limited by the risk of hemorrhagic transformation through breakdown of the blood-brain barrier (BBB). While cerebral ischemia itself causes increased permeability of the BBB, tPA exacerbates this by increasing the activation of matrix metalloproteinases (MMPs). The gelatinases, a subclass of MMP (MMP-2 and -9) are able to breakdown the structural proteins which provide structural integrity to the BBB, such as laminin, fibronectin and collagen.

The studies conducted in this thesis begin by outlining the temporal profile of MMP activation following tPA administration in a thromboembolic model of focal ischemia. Early activation of MMP-9 increases the loss of laminin and fibronectin surrounding cerebral blood vessels and allows IgG contained within vessels to extravasate to the surrounding parenchyma. The inhibition of MMP activation using doxycycline (DOXY) in combination with tPA thrombolysis was found here to exacerbate degradation of the BBB and increase infarct size.

Activation of MMPs requires cleavage of the pro-peptide domain to expose the active site and the catalytic Zn^{2+} ion. Activation of plasmin from its precursor plasminogen, by tPA, has been shown here to activate MMPs without cleavage of the pro-peptide domain. The gelatinases retain their proteolytic function and cannot be inhibited by DOXY, as the Zn^{2+} ion remains shielded by the pro-peptide domain.

This result is exclusive to DOXY as other Zn^{2+} chelating agents and MMP inhibitors were also tested in combination with tPA. This provides a mechanism for the increased severity of infarct found with the combination of tPA and DOXY.

Ischemic stroke primarily occurs in an elderly population yet the effects of tPA on an aged vasculature have not been previously investigated. With age there is a loss of structural protein expression and a loss of tight junction proteins which maintain the barrier properties of the BBB. MMP activation following cerebral ischemia which is further increased by tPA administration enhances the loss of BBB properties in an aged model of focal cerebral ischemia.

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Table of Contents

Chapter and Section	Page Number
Chapter 1	
Hemorrhagic Stroke.....	3
Ischemic Stroke.....	4
Global Ischemia.....	5
Focal Ischemia.....	7
Mechanisms of Cell Death.....	10
Necrosis.....	11
Apoptosis.....	13
Extrinsic Apoptotic Pathway.....	16
Intrinsic Apoptotic Pathway.....	17
Blood-Brain Barrier.....	19
Tissue Plasminogen Activator.....	21
Mechanisms of Action of tPA.....	24
Matrix Metalloproteinases.....	27
Regulation of MMPs.....	34
MMPs in Cerebral Ischemia.....	36
MMP inhibition.....	37
Tetracyclines.....	40
Downstream Signalling Effects of Tetracyclines.....	44
Angiogenesis – Involvement of MMPs and DOXY.....	46
Overall Objective.....	49

References.....	50
Chapter 2.....	67
Introduction.....	68
Materials and Methods.....	71
Focal Cerebral Ischemia.....	73
Measurement of Infarct Volume.....	74
Gelatin Zymography.....	74
IgG Extravasation.....	75
Immunohistochemistry.....	76
<i>In Situ</i> Zymography.....	77
Statistical Analyses.....	77
Results.....	78
Infarct Volume.....	78
Gelatin Zymography.....	80
IgG Extravasation.....	82
Immunohistochemistry – Laminin breakdown.....	85
Immunohistochemistry – Fibronectin breakdown.....	88
Immunohistochemistry – Co-localization.....	91
<i>In Situ</i> Zymography.....	93
Discussion.....	95
References.....	101
Chapter 3.....	104
Introduction.....	105

Materials and Methods.....	107
Animal Model.....	107
Tissue Preparation.....	108
Immunohistochemistry.....	108
Quantification of Neuronal Loss and Infarct Volume Measurements.....	109
Blood-Brain Barrier Breakdown.....	110
Laminin Immunohistochemistry.....	110
IgG Extravasation.....	111
Rat Brain Endothelial Cell Culture.....	111
Endothelial Cell Characterization.....	112
Experimental Protocol for Endothelial Cells.....	112
Gelatin Zymography.....	113
Gelatin Zymography of Conditioned Media.....	113
Statistical Analyses.....	114
Results.....	114
Quantification of Neuronal Loss and Infarct Volume.....	114
Blood-Brain Barrier Breakdown.....	120
Laminin Immunohistochemistry.....	120
IgG Extravasation.....	123
Cellular Localization of MMP-2 and -9.....	125
Gelatin Zymography.....	127
Gelatin Zymography of Conditioned Media.....	127

Discussion.....	130
References.....	134
Chapter 4.....	141
Introduction.....	142
Materials and Methods.....	146
Rat Brain Endothelial Cell Culture.....	146
Characterization of Endothelial Cells.....	147
Gelatin Zymography.....	148
Western Blotting.....	149
Results.....	149
Characterization of Endothelial Cells.....	149
MMP and VEGF Expression Changes with tPA Application.....	153
MMP Inhibition by DOXY Reduces VEGF Mobilization.....	154
VEGF Mobilization by MMP-9 is Plasmin-Independent.....	156
Discussion.....	158
References.....	161
Chapter 5.....	167
Introduction.....	168
Materials and Methods.....	170
Western Blot Analysis for BBB Components.....	170
Thromboembolic Ischemia.....	171
Fluoro-Jade Staining for Infarct Volume Measurement.....	171
Immunohistochemistry.....	172

IgG Extravasation.....	173
Gelatin Zymography.....	173
Statistical Analyses.....	173
Results.....	174
Western Blot for BBB Components.....	174
Fluoro-Jade.....	176
Laminin Immunohistochemistry.....	178
IgG Extravasation.....	180
Gelatin Zymography.....	182
Discussion.....	184
References.....	187
Chapter 6 – General Discussion.....	192
References.....	201

Table List

Table 1.1: MMP Substrate Specificity.....	29
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Figure List

Figure 1.1: Continuum of cell death.....	17
Figure 1.2: General MMP Structure.....	31
Figure 1.3: MMP Activation.....	34
Figure 1.4: Position of Constitutively Expressed MMP-2.....	36
Figure 1.5: General hydroxamic acid structure.....	40
Figure 1.6: Structure of the Tetracyclines.....	44
Figure 2.1: Schematic of experimental procedure.....	72
Figure 2.2: TTC infarct volume measurement.....	79
Figure 2.3: Gelatin zymography for MMP activation through the first 24 hours following focal cerebral ischemia.....	81
Figure 2.4: IgG Extravasation. Gross measure of BBB breakdown.....	82
Figure 2.5: Laminin Immunohistochemistry and histogram.....	86
Figure 2.6: Fibronectin Immunohistochemistry and histogram.....	89
Figure 2.7: Double immunofluorescent staining for the cellular localization of MMP expression.....	92
Figure 2.8: In Situ Zymography.....	94
Figure 3.1: Neuronal survival measurement.....	116
Figure 3.2: Infarct volume measurement.....	119
Figure 3.3: Laminin immunohistochemistry and histogram.....	122
Figure 3.4: IgG Extravasation.....	124
Figure 3.5: Double immunofluorescent staining for cellular localization of MMP expression.....	126

Figure 3.6: Gelatin zymography for MMP activation in brain homogenate and tPA-conditioned media.....	128
Figure 3.7: Gelatin Zymography of tPA-treated media.....	129
Figure 4.1: Proposed pathway.....	145
Figure 4.2: Endothelial cell characterization.....	151
Figure 4.3: MMP-9 activation is induced by tPA administration, leading to VEGF mobilization.....	152
Figure 4.4: MMP-9 inhibition with DOXY not MINO reduces VEGF mobilization....	155
Figure 4.5: Plasmin inhibition with α_2 -antiplasmin confirms that tPA can activate MMPs through a plasmin-independent mechanism.....	157
Figure 5.1: Western Blot analysis of tight junction proteins and laminin.....	175
Figure 5.2: Fluoro-Jade Quantification of Infarct Size.....	177
Figure 5.3: Laminin Immunohistochemistry.....	179
Figure 5.4: IgG Extravasation.....	181
Figure 5.5: Gelatin Zymography for MMP Activation.....	183
Figure 6.1: Schematic to summarize relationship between first three studies.....	196

ABBREVIATIONS:

ADP	adenosine diphosphate
AIF	apoptosis inducing factor
APAF-1	apoptosis activating factor-1
ATP	adenosine triphosphate
BBB	blood-brain barrier
Bcl	B-cell lymphoma
CAD	caspase-activated DNase
CMT	chemically-modified tetracyclines
CNS	central nervous system
CSF	cerebrospinal fluid
dATP	deoxyadenosine triphosphate
DSPA	desmodus rotundus plasminogen activator
DOXY	doxycycline
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
FADD	Fas receptor-associated death domain
FCI	focal cerebral ischemia
GCI	global cerebral ischemia
IAP	inhibitor of apoptosis protein
IL-1 β	interleukin 1 beta
LRP	low-density lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinase
MCA	middle cerebral artery
MINO	minocycline
MMP	matrix metalloproteinase
MT-MMP	membrane-type matrix metalloproteinase
NINDS	National Institute of Neurological Disorders and Stroke
NMDA	N-methyl D-aspartate
PAI-1	plasminogen activator inhibitor-1
PAR	protease activated receptor
PARP	poly (ADP-ribose) polymerase
PI3K	phosphatidylinositol 3-kinase
ROS	reactive oxygen species
SMAC	second mitochondria-derived activator of caspase
TIA	transient ischemic attack
TIMP	tissue inhibitor of metalloproteinase
TJP	tight junction proteins
tPA	tissue plasminogen activator
TNF α	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TRADD	tumor necrosis factor receptor-associated death domain
TTC	2,3,5-triphenyltetrazolium chloride
VEGF	vascular endothelial growth factor
ZO-1	zona occludens-1

Chapter 1:

Background and General Introduction

Overview

Each year in the United States, 200 000 deaths occur due to cerebrovascular disease (Harrison, 2005). The definition of cerebrovascular disease encompasses brain arterio-venous malformations, intracranial aneurysms, hemorrhagic stroke, and ischemic stroke. In Canada, over 50 000 strokes occur annually of which 15% are hemorrhagic in origin. Ischemic stroke is by far the most common form of stroke, occurring in the remaining 85% of stroke cases. Cerebral ischemia affects one person every 15 minutes (Canadian Stroke Network). Since 1952 the mortality rate of stroke has decreased by 70% (heartandstroke.ca). Treatment for ischemic stroke has therefore improved over the years but it by no means has an equivalent treatment potential to myocardial infarction, the ischemic equivalent in the heart. This thesis examines the downstream signaling of tissue plasminogen activator (tPA), currently the only Health Canada approved treatment for acute ischemic stroke (Canadian Stroke Network). Acute treatment with tPA for focal cerebral ischemia is associated with a risk in the transformation to a hemorrhagic situation. As a clot is lysed, it is possible that the structural integrity of the blood vessel becomes compromised. Therefore, it is important to begin with a brief discussion of hemorrhagic stroke before delving into the primary focus of this thesis which is ischemia. Following the discussion of stroke, I will describe the mechanisms of cell death that occur following a lack of blood flow to a region of the brain. As a stroke evolves, the blood-brain barrier (BBB) becomes compromised and as such, I have included a description of the intact BBB and how it can become increasingly permeable in the ischemic situation. The risk of hemorrhagic transformation associated with tPA

occurs through the compromise of the BBB, thus, this is followed by the function, benefits and drawbacks while also highlighting its role as an endogenous molecule. The conversion to hemorrhage from clot lysis is thought to be partially attributed to the activation of matrix metalloproteinases (MMPs). I conclude this overview by describing MMPs, their physiological importance and the implications following pathological activation. The MMPs and their proteolytic action tie all of the aforementioned themes in this thesis together. Following an ischemic event, MMPs become activated and this activation can be further exacerbated with the administration of tPA. The primary substrates of activated MMPs are the structural proteins of the extracellular matrix which contribute to the strength of the BBB. As a focus of this thesis is on the rescue of the BBB, I will also discuss the possibilities for MMP inhibition that have been studied to date with particular focus on the tetracycline derivatives, minocycline and doxycycline.

Hemorrhagic Stroke

Hemorrhagic stroke occurs when a cerebral blood vessel ruptures and there is bleeding into the brain parenchyma. This subtype of stroke has a much higher mortality rate than does ischemic stroke (Tejima et al., 2006) because beyond rehabilitative therapy there is little which can be done to reduce the amount of brain tissue affected. The ruptured vessel can either be within the brain or in the area surrounding it, as in the case of an epidural hemorrhage, subdural hemorrhage or a subarachnoid hemorrhage. Typically epidural, subdural and subarachnoid

hemorrhages are caused by head trauma or the rupture of an aneurysm but can also be attributed to extreme changes in blood pressure (Nestler et al., 2001). Tissue distal to the site of the hemorrhage is also affected as there is an increase in intracranial pressure associated with a hemorrhage. It is also possible for the hemorrhagic stroke to lead to the occlusion of a distal vessel. It is estimated that 30-40% of ischemic strokes will undergo a degree of hemorrhagic conversion (Tejima et al., 2006). To date, preventative therapy is the best tool that physicians have for hemorrhagic stroke (Adams et al., 2002).

To study potential therapies for hemorrhagic stroke, experimental animal models of intracranial hemorrhage are used. There are few hemorrhagic models in existence as it is quite difficult to emulate the hemorrhagic situation. Two models prevalent in the literature include (1), a collagenase model where collagenase is directly injected to the striatum disrupting cerebral microvasculature and (2), a whole blood model used to mimic a single large bleed that typically occurs in most clinical intracerebral hemorrhage cases (MacLellan et al., 2006). These models have illustrated a multifactorial involvement in the neuronal injury following hemorrhage. This includes, increased inflammatory cytokines, apoptosis, and activation of microglia (Tejima et al., 2006). Following an intracerebral hemorrhage, there is an ensuing increase in intracranial pressure that occurs within the first 48 hours, this can be a major cause of mortality (Tejima et al., 2006). Vasospasm, a condition in which blood vessels spasm causing vasoconstriction, can also occur following a hemorrhage, though this is typically associated with a subarachnoid hemorrhage

(Thiex et al., 2007). This type of vasoreactivity can cause local ischemia, resulting from the occurrence of a hemorrhage (Thiex et al., 2007).

Ischemic Stroke

The term stroke not only refers to a hemorrhagic event, but also an ischemic event, where a cerebral blood vessel is occluded. Ischemic stroke is the more common subtype of stroke with a prevalence of 85%. The occlusion of a blood vessel can be due to an embolus, thrombus or atherosclerosis (Nestler et al., 2001). An embolic stroke occurs when an occlusive substance such as fat, a fragment of bone, or a thrombus from a site distant to the area of the occlusion reaches the cerebral vasculature to occlude a smaller vessel. Embolic strokes are common in cases of recent heart surgery, a broken bone, or in situations where there is an abnormal heart rhythm (Harrison, 2005). Thrombotic stroke occurs when a vessel is occluded from the local formation of a thrombus making the occluding thrombus cerebral in origin. Narrowing of cerebral blood vessels can also occur; often due to an atherosclerotic lesion. The formation of which involves the deposition of plaques, cholesterol and white blood cells in the intima and inner media of cerebral arteries (Dorland, 2003). Ischemic strokes can be further subdivided based on the origin of the occlusion, either global or focal in nature.

Global Ischemia

As the term global implies, the entire brain is affected by a global ischemic event. Here, all peripheral sources of blood flow to the brain are interrupted. For example, this event can occur in cases of ventricular fibrillation or cardiac asystole (Harrison, 2005). Global insults are typically short-lived, lasting between 3 and 30 minutes, however, the cardinal feature of a global ischemic injury is a delay in cellular death (Lipton, 1999). Cell death may not occur for as many as 3-5 days after the interruption of blood flow (Lipton, 1999). Another cardinal feature is that, although the entire brain is universally deprived of oxygen and nutrients, the ensuing cell death is largely restricted to selected parts of hippocampus, the striatum, Purkinje cells in the cerebellum and neurons within the 3rd to 5th layers of cortex (Endo et al., 2006). These regions may be more susceptible to glutamate toxicity, calcium influx, activation of apoptosis, mitochondrial dysfunction and free radicals (Endo et al., 2006). The molecular mechanisms underlying this selective vulnerability remain unknown.

Animal models of global cerebral ischemia (GCI) are typically produced by the occlusion of more than one vessel rather than complete arrest of brain circulation (Lipton, 1999). Three commonly used *in vivo* GCI models used, the two-vessel occlusion in the gerbil, and a four-vessel or two-vessel occlusion combined with hypotension in the rat (Lipton, 1999). Whereas these models represent incomplete ischemia, complete ischemia refers to a situation where all arteries stemming from the heart are compressed, as in a neck-cuff model, or in cardiac arrest (Lipton, 1999). Here, blood flow to the brain is between 0 and 1% (Lipton, 1999). The gerbil model is the most commonly used model as it is much easier than rat models (Lipton,

1999). Gerbils are preferred for GCI models as this species does not have a complete Circle of Willis, in contrast to the rat, which has complete collateral cerebral circulation. The Circle of Willis is an anastomoses of arteries on the inferior aspect of the brain which provides redundancies in the cerebral circulation, such that if one artery were to become occluded, the area of brain supplied by this vessel would remain perfused (Ganong, 1999). Interestingly, in the case of GCI, following occlusion of the blood vessels, there is a rapid increase in the rate of blood flow to the brain which can be 2-3 times higher than at baseline levels. This increased rate of blood flow to the ischemic area can be seen as a compensatory mechanism to restore blood supply to ischemic areas. The compensatory increase in blood flow is followed by a reduction in blood flow to between 30 and 50% of baseline levels (Lipton, 1999), this is not known to contribute to the overall area of ischemic damage, but rather represents a reduced metabolic rate that can cause a reduction in processes utilizing ATP (Lipton, 1999).

Along with parenchymal changes which follow the occurrence of GCI, the BBB also becomes compromised. However, the increase in BBB permeability may not necessarily contribute to the extent of the GCI lesion. The permeability increases 6 hours after the insult, but there is no resulting extracellular edema. A more detailed discussion of the BBB can be found later in this chapter.

While GCI is fascinating for its selective vulnerability in different cell populations, the focus of this thesis is strictly within the realm of focal cerebral ischemia.

Focal Cerebral Ischemia

In the case of focal cerebral ischemia, a focused area of brain tissue is deprived of blood flow. Contributing factors to focal cerebral ischemia include a locally produced thrombus, an embolus originating from a distal site or an atherosclerotic lesion (Ganong, 1999; Nestler et al., 2001). Most commonly, it is the middle cerebral artery (MCA) which becomes occluded (Lipton, 1999) as blood flows through the carotid arteries to enter the brain, where there is a sudden narrowing of the vessels. Focal cerebral ischemia (FCI) can be transient or permanent depending on the nature of the occlusion. The degree of brain damage following a focal ischemic insult is much different than that seen in GCI. FCI does not result in a selectively vulnerable region of cell death, in the case of a focused area of reduced blood flow, a mass of brain tissue known as the infarct results reaching maximal size between 6 and 24 hours after the onset of the occlusion (Lipton, 1999). The infarct can increase in size in the days following this onset as this type of damage is more of an evolution. FCI is further differentiated from GCI in that even in the area immediately surrounding the occluded blood vessel, there is more often than not, a higher rate of blood flow than during GCI. There is also a gradient of damage extending outward from the core of the lesion (Lipton, 1999).

Numerous models of FCI exist and are widely used. Most commonly, one of the MCAs is targeted, either by physical occlusion as in a suture model or a thromboembolic model, or by the ligation of the branches on the superolateral surface of the cortex, known as a photothrombotic model. The physical occlusion

models involve insertion of a foreign material such as a small plastic suture sized to the vessel, and the suture can remain in place for permanent occlusion or can be removed in the case of a transient model of FCI. A thromboembolic model of FCI is a more clinically relevant model, in that this model induces the formation of a clot derived from the subjects' own blood. The blood is mixed with thrombin, one of the final mediators of the clotting cascade, causing the blood to clot. The clot is then reinjected and advanced to the opening of the MCA. Two important differences between GCI and FCI, are that (1) in FCI, even at the core of the insult, blood flow is always going to be higher than it is in GCI, and (2) the cell death that follows FCI is more of a gradation from the core extending to its outer boundary. This does not occur in the case of GCI, as mentioned, GCI causes cellular death and damage to selectively vulnerable areas of the brain such as the hippocampus. The core area of infarct is brain tissue immediately surrounding the occluded vessel. The core area of an infarct cannot be rescued from cellular demise. The area surrounding the infarct core is known as the ischemic penumbra (Green and Cross, 1997). Within the penumbra, cells are thought to undergo a more active form of cell death known as apoptosis to be discussed later in this section. In the case of a permanent occlusion of a cerebral blood vessel, both the core and penumbra will become infarcted. Depending on the strain of animal used and the model, there is always variation in the extent of the core and penumbral regions. Typically, and in contrast to a GCI insult, the infarct develops rapidly and continues to mature over several days. In the case of temporary FCI, the duration of MCA occlusion dictates the extent of the involvement of the affected regions. In permanent ischemia, the infarct is first noted

3-12 hours following occlusion. Damage due to a FCI insult is measured as the volume of infarct. Techniques used to measure the volume of infarct typically involve use of Nissl staining. Here, the stain will not be incorporated into a cell which does not have any nissl substance, cells which are dying or have died are unable to take up the stain. It is also possible to stain for active mitochondria, as with 2,3,5-triphenyltetrazolium chloride (TTC). Cells with compromised mitochondrial function are not capable of taking up the stain and so appear white in contrast to healthy tissue which appears red. Several markers of cellular dysfunction also exist and have been shown to parallel the expansion of the infarct indicating that these cells are indeed metabolically compromised. The progression of cell death in the penumbral regions is more delayed than within the core.

Neuroprotective agents have largely focused on reducing the spread of cell death within the penumbra. In so doing, compounds are specifically designed to target the progress of the cell death cascades.

Mechanisms of Cell Death

The brain has a very high requirement for oxygen and glucose and depends on oxidative phosphorylation for energy production (Dirnagl et al., 1999). The human brain on average consumes 3.5 mL of oxygen per 100 g of brain tissue per minute (Ganong, 1999). This represents approximately 20% of total body oxygen consumption (Ganong, 1999). As mentioned, there is a gradient of cellular demise that occurs surrounding the occluded vessel. At the core of the infarct, the tissue

immediately surrounding the occluded artery, most, if not all cells die. Blood flow in the core region is reduced to less than 15% of normal flow and in the penumbra, flow is reduced to less than 40% of normal (Lipton, 1999). Necrosis is the term often used to describe the passive form of cell death which is characteristic of this area (Ferrer and Planas, 2003).

Necrosis

Necrosis is characterized by a loss of cellular energy, and is morphologically manifested by cell and organelle swelling, rupture of the organelles, and finally in the bursting of the cell (Ferrer and Planas, 2003). Leakage of cellular contents to the extracellular space leads to increased toxicity involving neighbouring cells and can promote inflammation (Nestler et al., 2001). Necrosis allows uncontrolled function of proteases, calpains and the malfunction of proteins normally used for DNA repair which now become cell killers (Ferrer and Planas, 2003). Calpains breakdown cytoskeletal proteins. While proteases such as phospholipases, can target membrane phospholipids and alter membrane integrity. Free radicals which are largely activated during necrosis are molecules with an unpaired electron in their outer shell, causing the molecule to be highly reactive and unstable (Nestler et al., 2001). Free radicals can disrupt membrane integrity by peroxidizing membrane fatty acids or can cause strand breaks in DNA (Nestler et al., 2001; Lipton, 1999).

Loss of cellular energy occurs due to a lack of oxygen and glucose required for the production of adenosine triphosphate (ATP). When blood flow ceases, the

electron transport chain is stopped and oxidative phosphorylation, the primary source of ATP production in the brain, cannot occur (Lipton, 1999). Without ATP, Na^+ pumps are unable to maintain the low levels of intracellular Na^+ necessary for cellular homeostasis. As a result, Na^+ enters the cell, along its concentration gradient. This influx of Na^+ would typically signal a depolarization however, with the immense influx of Na^+ , there is a massive abnormal membrane depolarization. This depolarization causes the release of glutamate, an increase in intracellular calcium (Ca^{2+}) and further depletion of ATP (Lipton, 1999). It was once thought that use of Na^+ -channel blockers if administered early enough following vessel occlusion, could be neuroprotective. However, this has not proven true, as the loss of ATP production exceeds the capacity of ATP production by oxidative phosphorylation.

Calcium (Ca^{2+}) is another key ionic gradient which, when not maintained, can be highly toxic to cells. Ca^{2+} is very tightly regulated within cells as it is involved in the activation of several downstream enzymatic and biochemical pathways. These pathways regulate most physiological processes including, synaptic plasticity, secretion, and contraction, to name a few. Normal Ca^{2+} influx occurs through two different channels, voltage-gated Ca^{2+} channels which are opened by a depolarizing stimulus, and ligand-gated channels, activated by neurotransmitter release. In an ischemic situation, with a loss of ATP, there is an inability to clear the excess release of intracellular Ca^{2+} . A massive influx of Ca^{2+} ensues, activating numerous second messenger systems rendering many cellular processes unmanageable by the cell (Dirnagl et al., 1999; Nestler et al., 2001). The energy-

dependent processes that would normally resolve the influx of Ca^{2+} , such as reuptake of excitatory amino acids are disrupted causing further accumulation of extracellular glutamate by continuous signaling of cellular activity. The primary excitatory amino acid in the central nervous system is glutamate. A perpetual cycle of glutamate receptor activation, calcium influx and water entering the cell begins as the initial release of glutamate can trigger neighbouring cells to depolarize, involving more cells in this cycle. The neural damage that occurs by glutamate overactivity can be a result of the failure of reuptake mechanisms, which are ATP-dependent. This process is referred to as excitotoxicity (Lo et al., 2004b). Excitotoxicity can also occur by means other than increased Ca^{2+} through glutamate receptor-coupled channels, such as through acidosis, the accumulation of lactate due to the absence of aerobic respiration resulting in the formation of free radicals (Lo et al., 2004b).

As necrosis is a dissipative process with damage attributed to a loss of ATP and a loss of ionic gradients (Lipton, 1999) there has been little success in the development of neuroprotective agents developed to target necrotic cell death.

The mode of cell death depends primarily on the severity of the initiating injury. In the case of cerebral ischemia, this could be defined as the length of time that a cerebral blood vessel is occluded, the extent of the reperfusion injury, or the size of the thrombus in the affected area. As mentioned, necrosis is primarily observed in the core area of an infarct. In tissue distal to the occluded vessel, there is a shift in the type of cell death which occurs from necrosis to delayed, DNA-mediated cell death or apoptosis (Lipton, 1999).

Apoptosis

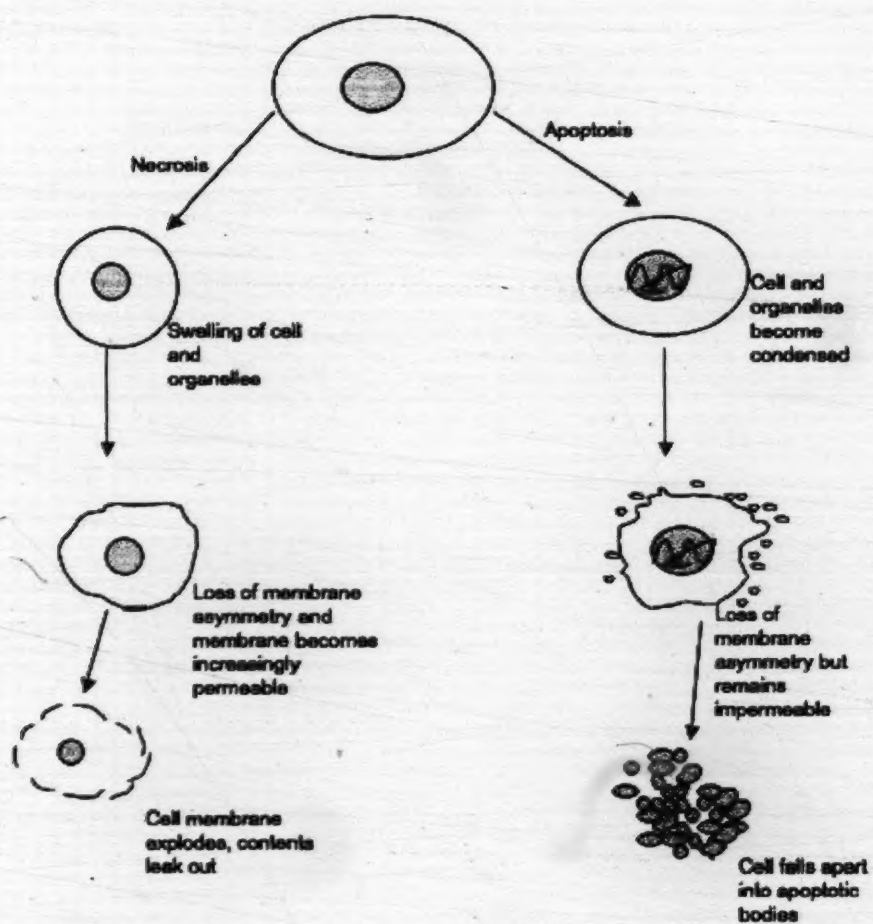
The delineation between necrotic cells and cells that have undergone apoptosis is not distinct, necrosis and apoptosis can be thought to exist along a continuum (as depicted in Figure 1.1). This figure depicts necrosis occurring when active transport mechanisms fail and there is a loss of the ability to maintain ionic gradients across a cell membrane. Apoptosis may represent a more moderate cellular injury, or perhaps a short-lived ischemic event in which ionic gradients are maintained yet the balance between pro-survival protein expression and pro-apoptotic protein expression is shifted toward the induction of apoptosis (Lipton, 1999; Nestler et al., 2001).

The area surrounding the infarct core is known as the ischemic penumbra (Green and Cross, 1997). This penumbral area is hypo-perfused and cells here may undergo an active form of cell death, one that requires protein synthesis (Ferrer and Planas, 2003; Paschen, 2003). The apoptotic cascade has been widely studied and is a primary target in the development of neuroprotective agents (Cardone et al., 1998; Dirnagl et al., 1999; Ferrer and Planas, 2003; Paschen, 2003; Beere, 2004). Blood flow to the penumbra is insufficient to maintain normal electrical activity but does preserve the function of ion channels (Ferrer and Planas, 2003; Paschen, 2003). The classic morphological identifiers of apoptosis include DNA fragmentation, chromatin condensation, cytochrome c release from mitochondria, and membrane blebbing (Namura et al., 1998; Paschen, 2003).

A key identifier of apoptosis is the activation of caspases. Caspases are a family of cysteine proteases that translocate to the cell nucleus and can commit a cell to die (Riedl and Shi, 2004). All caspases are produced in the cell as inactive zymogens (Riedl and Shi, 2004), and are cleaved either by other caspases or other proteases to render them active. Once active, they can cleave any number of downstream substrates. Caspases are typically divided into one of two categories: the initiator caspases including caspase-2, -8, -9 and -10; and the effector caspases, which include caspase-3, -6 and -7 (Riedl and Shi, 2004). Effector caspases are normally activated by an initiator caspase, whereas initiator caspases are auto-activated and may require the formation of a complex with other molecules (Riedl and Shi, 2004). The high regulation of initiator caspases is necessary as the downstream effects of caspase activation results in proteolytic cleavage of a broad spectrum of targets (Riedl and Shi, 2004). Apoptosis, is a highly regulated process that can be inhibited by a group of natural inhibitors of apoptosis (IAPs). IAPs prevent the activation of pro-caspases and inhibit the enzymatic activity of active caspases (Chan, 2004; Philchenkov, 2004). However once apoptosis has been initiated, in this case, through the permeabilization of the mitochondrial membrane, pro-apoptotic factors are released to the cytosol. These pro-apoptotic factors can include the Second Mitochondria-derived Activator of active Caspases (Smac/DIABLO). Smac/DIABLO binds to IAPs inhibiting the anti-death function of the IAP family (Philchenkov, 2004). The involvement of pro-apoptotic molecules is thought to be largely regulated by reactive oxygen species (ROS) and the oxidative stress that a cell is typically under following an ischemic event (Chan, 2004).

Several exogenous inhibitors of caspases have been developed in an attempt to halt cell death. These have not proven to be very neuroprotective, due to the timing of caspase activation. Inhibiting activation of caspases may rescue cells which could result in a non-functional cell.

Figure 1.1. Continuum of cell death. Necrosis on the left and apoptotic cell death on the right. The major morphological changes are featured here.



Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway involves members of the death receptor family such as the Fas receptor, and the tumor necrosis factor receptor (TNFR) (Chan, 2004). Here, a Fas ligand binds to the Fas receptor and an adaptor molecule, the Fas receptor-associated death domain (FADD) (Chan, 2004). Receptor binding activates procaspase-8 to caspase-8 which goes on to activate caspase-3, this entire process occurs independently of the mitochondria (Dirnagl et al., 1999; Chan, 2004). Activation of caspase-3 initiates the cleavage of poly-(ADP-ribose)-polymerase (PARP), leading to the activation of caspase-activated DNase causing the classically observed DNA laddering characteristic of apoptotic cells (Chan, 2004). PARP is a nuclear matrix protein typically activated by peroxynitrite-induced DNA strand breaks, which transfers ADP ribose moieties from NAD⁺ thus repairing the strand break. In cases of excess PARP cleavage as in an ischemic situation, NAD⁺, its substrate, are depleted. ATP is required for the synthesis of NAD⁺, thus in an attempt to create more NAD⁺, ATP is further depleted (Kwan et al., 2004).

Intrinsic Apoptotic Pathway

In contrast to apoptosis induced by extrinsic signals is the intrinsic apoptotic pathway which involves the mitochondria. Here, a mitochondrial pore is formed resulting in the release of cytochrome c and apoptosis activating factor-1 (APAF-1) into the cytosol (Philchenkov, 2004). Cytochrome c interacts with APAF-1, caspase-9, and deoxyadenosine triphosphate (dATP) to form a supramolecular complex known as the apoptosome (Chan, 2004; Fumarola and Guidotta, 2004; Philchenkov, 2004). This apoptosome complex activates caspase-3, the primary effector caspase.

Caspases downstream of caspase-3, as mentioned, cleave various substrate proteins such as PARP ultimately causing the cell to die (Chan, 2004).

The B cell lymphoma (Bcl) family of proteins is also involved in the fate of neurons, having both pro-apoptotic and pro-survival members (Lipton, 1999; Nestler et al., 2001). For example, Bax, a pro-apoptotic molecule, dimerizes within the mitochondrial membrane and enhances the release of cytochrome c (Nestler et al., 2001). In contrast, Bcl-2, a pro-survival family member, interferes with Bax thus inhibiting cytochrome c release (Nestler et al., 2001).

In addition to the activation of caspases, apoptosis can be caspase-independent this involves apoptosis-inducing factor (AIF). AIF is released from the mitochondrial intermembrane space to the cytosol where it translocates to the nucleus causing chromatin condensation and DNA fragmentation (Dirnagl et al., 1999). In a situation of oxidative stress there is an imbalance between antioxidant mechanisms and free radical production. The increased production of free radical molecules can result in the loss of mitochondrial integrity that can lead to apoptosis.

The blood-brain barrier (BBB) protects brain tissue from the infiltration of an inflammatory response. However, in an ischemic situation, the BBB is compromised and pro-inflammatory molecules are able to promote inflammation within the brain further contributing to the process of cell death (Danton and Dietrich, 2003).

Blood-Brain Barrier

The BBB is a physical barrier separating circulating blood from the brain parenchyma (Gaillard et al., 2001; Jodoin et al., 2003; Ma et al., 2005; Schiera et al., 2005). This barrier is central to the maintenance of brain homeostasis as proteins are prevented from entering the brain and the penetration of smaller molecules is greatly slowed (Abbott et al., 1992; Schiera et al., 2005; Chan et al., 2006). Water, CO₂, and O₂ cross the BBB quite easily while glucose, the major energy source for the brain, crosses slowly but passively (Ganong, 1999). There is relatively little vesicular transport across cerebral blood vessels although many carrier-mediated transport systems do exist. These systems serve to protect the brain from toxic substances (Ganong, 1999). The reverse movement from brain parenchyma to the blood is quite different from the flow of blood to brain, in that it is freer due to the bulk flow of cerebrospinal fluid (CSF) into venous blood (Ganong, 1999). Throughout the brain, there are exceptions to this restrictive BBB; these areas are known as circumventricular organs, they include the posterior pituitary gland, the area postrema, the lamina terminalis and the subfornical organ (Ganong, 1999). The capillaries in these areas are fenestrated allowing otherwise restricted molecules to cross into the brain parenchyma (Ganong, 1999). For example, the area postrema acts as a chemoreceptor trigger zone able to trigger the vomit reflex in response to chemical changes in blood plasma (Ganong, 1999). Fenestrated regions sample circulating blood to ensure the maintenance of homeostasis (Ganong, 1999).

In areas of the BBB which are not fenestrated and do not freely allow the passage of molecules, there are tight junction proteins (TJPs) between the endothelial cells that line cerebral capillaries (Fletcher et al., 2006). These tight junctions are

further reinforced by perivascular astrocytic endfeet apposed to the endothelial cells (Fletcher et al., 2006). Structurally, TJPs form a continuous network of parallel, interconnected intramembrane strands (Schiera et al., 2005; Fletcher et al., 2006). Several TJPs have been identified including, zona-occludens-1 (ZO-1), occludin and the claudin family (Farshori and Kachar, 1999). Occludin, an integral membrane protein together with the claudin family, may be directly involved in the formation and resealing of tight junctions (Farshori and Kachar, 1999). Under normal, physiological conditions, tight junctions form an almost impenetrable barrier preventing the passage of all but the smallest hydrophilic molecules (Cleaver and Melton, 2003; Fletcher et al., 2006). As the BBB integrity is lost, inflammatory cells and fluid can penetrate the brain thus contributing to edema and cell death (Kamada et al., 2007). Breakdown of the BBB begins as a loosening of the TJPs and withdrawal of perivascular astrocyte end-feet which allows the passage of circulating pro-inflammatory molecules, proteases, and adhesion molecules. Following an ischemic injury, opening of the BBB has been shown to occur in a biphasic manner. The biphasic opening of the BBB corresponds to an initial acute disruption 3-5 hours following the occlusion and later, a further increase in BBB permeability occurs about 48 hours after ischemic onset (Valable et al., 2005). The later, secondary opening may be due to increased expression of vascular endothelial growth factor (VEGF) (Zhang et al., 2000). MCA occlusion can evoke the expression of VEGF, and this may be involved in angiogenesis (Zhang et al., 2000). While the increase in angiogenesis is beneficial to the ischemic penumbra in returning blood flow to an otherwise ischemic area, it may also be harmful by enhancing BBB permeability

through the promotion of the proliferation and migration of endothelial cells which can in turn lead to hemorrhagic transformation (Zhang et al., 2000; Valable et al., 2005).

While the biphasic permeability of the BBB may be destructive or beneficial with respect to the ischemic lesion and endogenous recovery processes, the administration of the clot lysis agent, tissue plasminogen activator (tPA) can further contribute to the increased permeability of the BBB following cerebral ischemia.

Tissue Plasminogen Activator

As most ischemic strokes are thromboembolic in origin, restoration of blood supply is the only strategy for the medical management of stroke (Adams et al., 2002). Three agents commonly used are, plasminogen activators to lyse an existing clot, anticoagulants and anti-platelet aggregating agents to prevent further clotting (Adams et al., 2002). The role of anticoagulant and anti-platelet aggregating medications have not been defined in the acute ischemic stroke situation, however, for the secondary prevention and reduction of risk in high-risk patients, these medications have proven effective (Amano et al., 2001). Only thrombolysis with tPA is recognized as clinically effective for improving the outcome of acute stroke patients (Adams et al., 2002).

Rapid restoration of blood flow through timely thrombolysis of a cerebral occlusion is an absolute requirement for the treatment of acute ischemic stroke.

Tissue plasminogen activator (tPA) converts the zymogen, plasminogen, to the active plasmin which is able to cleave fibrin bonds of an existing clot (Amano et al., 2001; Benchenane et al., 2005a). Approval of the use of tPA for focal cerebral ischemia follows from the success of tPA in the ischemic myocardium. With a focus on clot lysis, many plasminogen activators have been investigated for their potential clinical use including streptokinase however, this failed in three separate clinical trials for the treatment of acute stroke, due to increased mortality caused by massive brain hemorrhage (Amano et al., 2001; Sheehan and Tsirka, 2005). Other thrombolytic agents which have been tested clinically include, staphylokinase which is significantly more fibrin-specific than tPA and has been shown to be at least as effective as tPA in lysing arterial emboli (Kaur et al., 2004). In a rat model of FCI, staphylokinase, streptokinase, and tPA were directly compared and the three demonstrated a dose-related extension of the focal injury (Nagai et al., 1999). This association of tPA with hemorrhagic transformation has led to the development of third generation thrombolytic agents. These agents have longer half-lives and greater penetration to the thrombus matrix (Kaur et al., 2004). For example, tenecteplase has been genetically modified resulting in an extended half-life and improved fibrin-specificity and it has an increased resistance to plasminogen activator inhibitor-1 (PAI-1) (Kaur et al., 2004). Desmotopase, or desmodus rotundus plasminogen activator (DSPA), is a vampire bat salivary plasminogen activator studied and compared to tPA in animal models of cerebral ischemia, may be less toxic to neuronal cells than tPA (Grandjean et al., 2004; Kaur et al., 2004;

Reddrop et al., 2005; Lopez-Atalaya et al., 2007). Phase III clinical trials are underway (Lopez-Atalaya et al., 2007).

There is a narrow window of opportunity for the benefit of thrombolysis by tPA. Beyond three hours, the risk of hemorrhagic transformation is greatly increased (Group, 1995; Amano et al., 2001) and the risk of increased bleeding beyond this time point is well-documented (Group, 1995). Two studies have examined the use of tPA 6 hours after ischemic onset in animal models, and both have demonstrated a high rate of brain hemorrhage when treated after this time (Amano et al., 2001; Weintraub, 2006). Thus, the timing of tPA administration and patient selectivity criteria are critical to the beneficial outcome of acute stroke. Specified criteria for tPA administration include normal blood pressure, no pre-existing vascular problems including diabetes, no history of heart problems and finally, a young age, defined as less than 80 years (Sylaja et al., 2006). Despite strict adherence to protocol, approximately 1 in 16 patients will develop a brain hemorrhage following tPA administration (Group, 1995; Green and Cross, 1997; Kaur et al., 2004; Weintraub, 2006).

Mechanism of Action of tPA

Endogenous tPA is highly regulated by its natural inhibitor, plasminogen activator inhibitor-1 (PAI-1; Zhuo et al., 2000), as a cell-signaling molecule in neurons and glia the signaling effects of tPA do not seem to require proteolytic activity (Lo et al., 2004a). The cleavage product of tPA, plasmin, is able to bind to protease-activated receptors (PARs) (Junge et al., 2003). This binding of plasmin to PAR-1, has been shown to be an important interaction in neurodegeneration (Junge

et al., 2003). PAR-1 is expressed on endothelial cells as well as on smooth muscle cells and activation of PAR-1 can contribute to the potentiation of NMDA receptors which can exacerbate ischemic damage (Junge et al., 2003).

Degradation of the extracellular matrix is the current hypothesis for the mechanism through which the tPA/plasmin system promotes neurodegeneration (Lo et al., 2002; Benchenane et al., 2004; Lo et al., 2004a; Benchenane et al., 2005a). It is implied that plasmin destroys the layer upon which neurons are laid and form their network causing a form of cell death known as anoikis (Lo et al., 2002; Benchenane et al., 2004; Lo et al., 2004a; Benchenane et al., 2005a). Anoikis is a subtype of apoptosis in which anchor-dependent cells are released from their extracellular matrix and undergo regulated cell death (Lee and Lo, 2004). Recently, it has been shown that tPA can potentiate signaling mediated by glutamatergic receptors by proteolytically regulating NMDA receptors and increasing the influx of Ca^{2+} (Pfefferkorn and Rosenberg, 2003). The mechanism suggested for this neurotoxicity involves cleavage of the NMDA NR1 subunit, causing the NMDA receptor to be more sensitive to glutamate (Pfefferkorn and Rosenberg, 2003).

Several *in vivo* and *in vitro* studies have demonstrated the cellular toxicity of exogenously administered tPA (Kaur et al., 2004), and knockout studies have demonstrated the neuroprotective potential of ridding the CNS of tPA entirely (Kaur et al., 2004). Mice genetically modified to have lost tPA have been shown to be resistant to excitotoxic cell death induced by administration of kainate (Kaur et al., 2004; Benchenane et al., 2005b).

The primary action of exogenously applied tPA, plasmin break down of fibrin-rich clots, occurs within blood vessels (Lo et al., 2004a). It has been recently shown that minimal amounts of exogenous tPA are able to cross an intact BBB, but will flood into the brain parenchyma in the case of a compromised BBB, causing further toxicity beyond the ischemic insult (Benchenane et al., 2005a). The intention of treatment with tPA is to restore blood flow to an ischemic area. However, since the first tPA trial performed by the National Institute of Neurological Disorders and Stroke (NINDS), the observed risk of hemorrhagic transformation has tainted the potential benefits of tPA use (trial, 1995; Dijkhuizen et al., 2001; Benchenane et al., 2004; Weintraub, 2006). The destructive capabilities of exogenous tPA to the extracellular matrix are highlighted in the ischemic situation as the BBB has become compromised. Matrix destruction is thought to occur primarily through the proteolytic action of matrix metalloproteinases (MMPs). MMPs can be beneficial in angiogenesis and the restoration of collateral circulation in ischemic areas (Bergers et al., 2000; Karagiannis and Popel, 2006; Lee et al., 2006b).

While exogenously applied tPA in a non-ischemic paradigm has not been found to induce BBB breakdown, in a pathological situation tPA can exacerbate excitotoxic injury, peripheral nerve injury and Wallerian degeneration (Kaur et al., 2004; Benchenane et al., 2005b; Benchenane et al., 2005a). It is possible exogenously applied tPA can exacerbate lethal cellular signaling in the presence of ischemia (Benchenane et al., 2005a). The application of tPA to an already compromised BBB may allow Wallerian degeneration and nerve injury to occur at a faster rate thus with more dangerous consequences (Kaur et al., 2004; Benchenane et

al., 2005a). The increased rate of neural degeneration was further verified in a study examining the early application of tPA following cerebral ischemia (Benchenane et al., 2005a). In this particular study, tPA was administered very soon following an ischemic injury prior to the destruction of the BBB (Benchenane et al., 2005a). This early administration of exogenous tPA was still able to cross the intact BBB, suggesting the presence of a receptor-mediated transport mechanism (Benchenane et al., 2005a). The low-density lipoprotein receptor-related protein (LRP) system has been proposed to allow the passage of exogenous tPA through an intact BBB, albeit at a slower rate than if the BBB is permeable (Benchenane et al., 2005a). However, sham-operated animals receiving tPA, used as controls in the experiments conducted throughout this thesis, did not demonstrate any evidence for increased tPA in an uninjured brain. LRP-mediated transport of tPA across an intact BBB remains under investigation. Regardless of the mechanism of passage of tPA across the BBB, tPA is an upstream effector of several cellular cascades. Of these cascades, the involvement of MMPs are of great interest, particularly for their involvement in breakdown of the BBB.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-binding proteolytic enzymes able to degrade the extracellular matrix² (Crocker et al., 2006). In a physiological setting, MMPs make way for new tissue, as in angiogenesis, bone remodeling, wound healing and neurogenesis (Wee Yong et al., 2001; Crul et al.,

2002; Gu et al., 2002; Sanceau et al., 2002; Wang et al., 2006). The ability of MMPs to breakdown components of the extracellular matrix, specifically targeting laminin, collagen IV, and fibronectin (Montaner et al., 2003), renders it particularly destructive in a pathological setting. Abnormal MMP activity has been linked to multiple sclerosis, Alzheimer's disease, cancer and stroke (Wee Yong et al., 2001; Crocker et al., 2006). The effects of MMPs extend beyond simply cleaving extracellular matrix proteins as this group of proteases has also been found to cleave growth factor receptors, receptor tyrosine kinases and other proteases (Aitken et al., 2006).

MMPs have been classified according to their substrate specificity (details are included in Table 1.1) there are also membrane-type MMPs (MT-MMPs) of which eight have been described (Wee Yong et al., 2001; Montaner et al., 2003; Lalu et al., 2005).

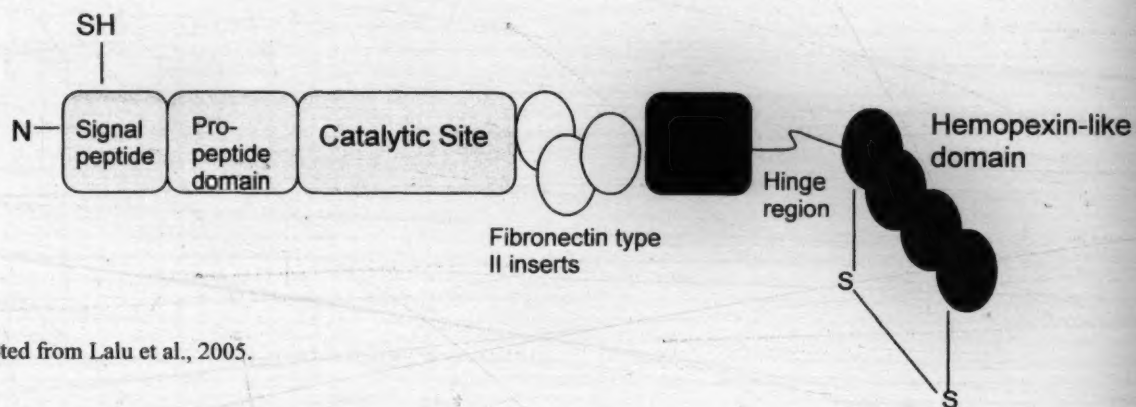
Table 1.1. Substrate specificity of MMPs.

Family Name	MMP	Pro-MMP Substrate	Matrix Substrate
Collagenases	1, 8, 13, 18	2, 9	Collagen, gelatin, proteoglycan, aggrecan
Gelatinases	2, 9	1, 9, 13	Collagen, gelatin, fibronectin, laminin, vitronectin, aggrecan, proteoglycan, elastin
Stromelysins	3, 10, 11	1, 7, 8, 9, 13	Collagen, aggrecan, elastin, fibronectin, laminin, proteoglycan, gelatin, vitronectin
Matrilysin	7	1, 2, 9	Collagen, aggrecan, casein, elastin, fibronectin, laminin, proteoglycan, gelatin, vitronectin
MT-MMPs	14, 15, 16, 17, 24, 25	2, 13	Collagen, gelatin, fibronectin, laminin, elastin, fibrinogen/fibrin

Adapted from Wee Yong et al., 2001. Nature Reviews Neuroscience

All MMPs are structurally related (Wee Yong et al., 2001) and include an amino-terminal signaling peptide allowing for secretion into the endoplasmic reticulum and transport out of the cell (Wee Yong et al., 2001; Sanceau et al., 2002; Lalu et al., 2005). MMPs all have a propeptide region shielding the catalytic domain next to it. The catalytic domain has a zinc-binding motif (Lalu et al., 2005). At the carboxy-terminus, a hemopexin domain confers substrate specificity and allows for docking with other proteins (Wee Yong et al., 2001; Sanceau et al., 2002; Lalu et al., 2005). This hemopexin domain contains four repeat units, with the first and last being connected by a disulphide bridge (Wee Yong et al., 2001; Gu et al., 2002). The carboxy- and amino-termini are connected by a short hinge region which can vary in length based on the type of MMP (Wee Yong et al., 2001). In its zymogen form, the catalytic Zn^{2+} ion is coordinated to a cysteinyl sulphhydryl group on the propeptide domain and is thus inactive (Wee Yong et al., 2001; Lalu et al., 2005).

Figure 1.2. General MMP Structure. At the N-terminus, MMPs have a signal peptide, which allows for transport out of the cell. The pro-peptide domain has a free thiol group for Zn^{2+} -binding, the domain is hydrophobic and shields the catalytic site. The catalytic domain is also referred to as a matrixin-fold. The fold forms substrate-binding pockets and coordinates with the catalytic Zn^{2+} . At the C-terminus, most MMPs have a hemopexin-like domain, this allows for substrate specificity.



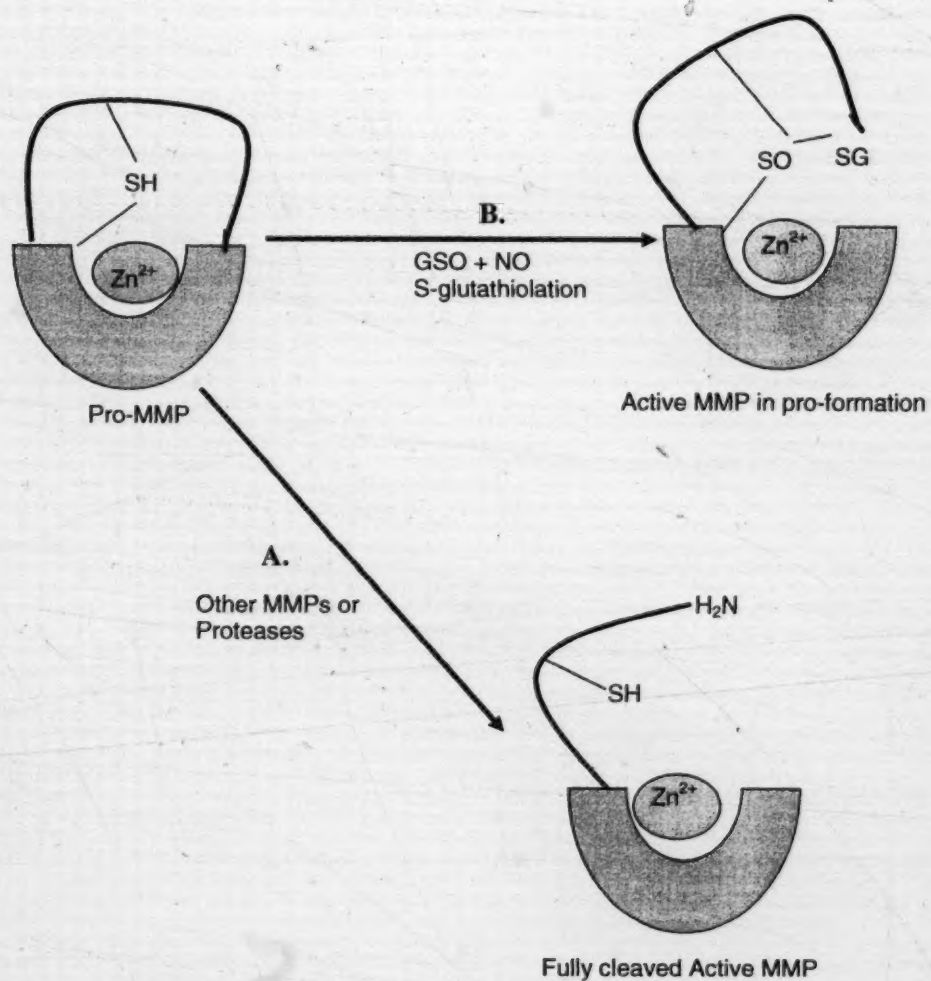
Adapted from Lalu et al., 2005.

The MT-MMPs contain a transmembrane domain at the carboxy-terminus (Wee Yong et al., 2001). These MMPs are activated intracellularly and are then inserted to the cell membrane (Lalu et al., 2005). Some MMPs have been shown to have slightly differing structural features. Matrilysin, for example, lacks a hemopexin domain, while the gelatinases have an extra fibronectin-like domain which confers their specificity for extracellular matrix proteins (Cuzner and Opdenakker, 1999a).

To become activated, MMPs expose the catalytic Zn^{2+} ion. There are four possible mechanisms to this activation. First, a stepwise activation in the extracellular space, with cleavage of the vulnerable loop in the propeptide domain, the prodomain structure breaks down and the shielding of the catalytic cleft is withdrawn (Kwan et al., 2004; Lalu et al., 2005). Secondly, proteolytic cleavage could occur at the cell surface and the active MMP released from the cell, allowing for increased MMP activity in the immediate pericellular space (Lee et al., 2006b). Cell surface activation is likely due to cleavage by the MT-MMPs. The third possible mechanism of activation, occurs in MT-MMPs, where they are activated intracellularly prior to insertion to the cell membrane (Lalu et al., 2005). Finally, it is possible for MMPs to be activated by oxidative stress (Okamoto et al., 2001; Lalu et al., 2005). This pathway to activation can occur either intracellularly or extracellularly (Okamoto et al., 2001; Lalu et al., 2005). Here, endogenous oxidants such as, superoxide anion, hydrogen peroxide or peroxynitrite, can oxidize the sulphhydryl bond of the cysteinyl group which binds to the catalytic Zn^{2+} ion. The disruption of this inhibitory bond allows for hydration of the catalytic site, however the propeptide domain is not removed (Lalu et al., 2005). This process has come to

be known as S-glutathiolation, with a disulfide S-oxide formation leading an almost undetectable change in molecular weight and resulting in an active pro-form of MMP (Okamoto et al., 2001; Gu et al., 2002; Kwan et al., 2004; Lalu et al., 2005). This oxidative pathway to activation of MMPs has been demonstrated in MMP-1, -2, -8 and -9 (Lalu et al., 2005). In a similar fashion, nitric oxide has been shown to activate MMPs by reacting with the cysteine thiol in a process known as S-nitrosylation (Gu et al., 2002). The cysteine reactivity with nitrosylating agents such as nitric oxide requires that there are basic and acidic residues flanking the reactive cysteine; this requirement is met in the structure of MMPs (Gu et al., 2002). Nitrosothiols can be short-lived and their reaction can be reversed by reducing agents. Formation of the active pro-MMP can also lead to irreversible oxidative reactions which would permanently activate MMPs, thus resulting in further BBB breakdown (Gu et al., 2002). The traditional and oxidative mechanisms of MMP activation are depicted in Figure 1.3.

Figure 1.3. MMP Activation. The uppermost black line represents the pro-peptide region, shielding the active site and Zn^{2+} ion. A. Depicts the traditional mode of MMP activation in which another MMP or an upstream protease cleaves the pro-peptide domain, allowing it to withdraw. Once withdrawn, the catalytic site is exposed and the MMP is rendered active. B. Depicts MMP activation in the presence of nitrosylating agents such as peroxynitrite or thiolating agents as in glutathione. Here, the pro-peptide region is not removed, and the catalytic site remains shielded, though the MMP retains all proteolytic activity.

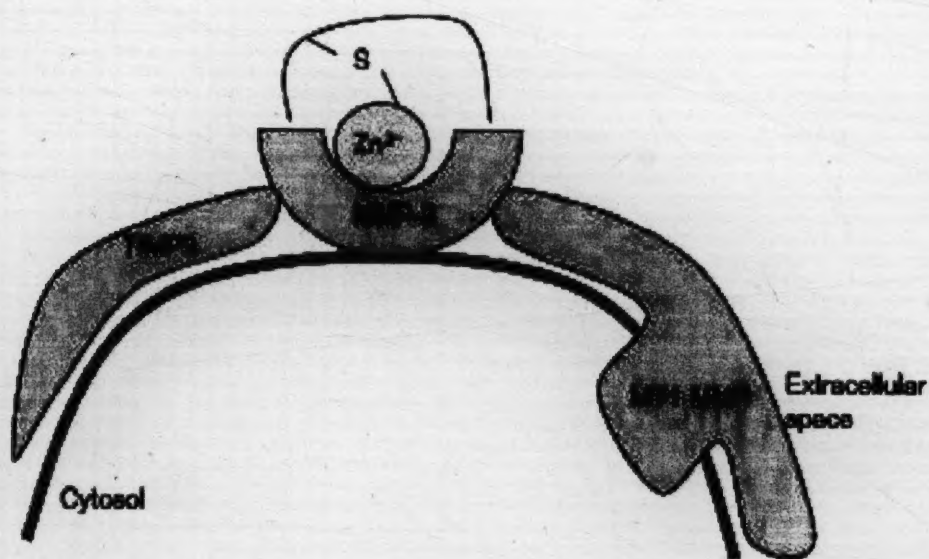


Regulation of MMPs

MMPs are tightly regulated due to their ability to cause widespread destruction of the extracellular matrix. Most MMPs are not constitutively expressed and are regulated primarily at the level of transcription. Transcription of MMPs can be promoted by inflammatory cytokines as well as growth factors, and oncogenes (Wee Yong et al., 2001; Sanceau et al., 2002). Tissue inhibitors of metalloproteinases (TIMPs) represent another level of MMP regulation. TIMPs render MMPs inactive by binding directly to the active site (Wee Yong et al., 2001; Sanceau et al., 2002). Four TIMPs have been identified thus far and each binds an MMP in a 1:1 stoichiometric ratio (Qi et al., 2003; Lalu et al., 2005). Generally the TIMPs do not demonstrate a high specificity for any one MMP however, TIMP-2 is known to preferentially bind MMP-2 and TIMP-1 to bind MMP-9 (Lalu et al., 2005). During the induction of cellular proliferation and migration there must be a shift in the relative protein levels of MMPs and TIMPs (Lamoreaux et al., 1998).

The gelatinases, MMP-2 and MMP-9, also known as gelatinase A and B respectively, have been highly implicated in BBB destruction which occurs following focal cerebral ischemia due to their specificity to degrade laminin, fibronectin, and collagen (Montaner et al., 2003; Campbell et al., 2004). Much stroke research has focused primarily on the activation and inhibition of the gelatinases due to their dominant role in breaking down the BBB. Interestingly, MMP-2, gelatinase A, is one MMP which is constitutively expressed (see Figure 1.4 for further details).

Figure 1.4. Position of constitutively present MMP-2. In a quiescent state, MMP-2 in its pro-form exists on the extracellular membrane tethered between TIMP-2 and MT1-MMP. This dual form of regulation presumably makes MMP-2 much more difficult to become activated.



MMPs in Cerebral Ischemia

Following an acute focal cerebral ischemic event it has been shown that MMP-2 and -9 play central roles in the proteolytic degradation of the BBB (Montaner et al., 2003; Kamada et al., 2007). Their increased activation in the hours to days following an ischemic event is related to the development of edema and hemorrhagic transformation (Montaner et al., 2003; Rosell et al., 2006). In fact, in human studies, elevated MMP-9 levels have been shown to correlate with poor neurological outcome (Rosell et al., 2006). The devastating effects of increased expression and activation of the gelatinases following cerebral ischemia are well recognized and this can be attributed to their primary specificity for proteins of the ECM (Clark et al., 1997; Campbell and Pagenstecher, 1999; Asahi et al., 2000; Aoki et al., 2002; Bjornsson et al., 2004). Recently, it has been shown that MMP-9 can also degrade TJPs such as zona occludens-1 (Yang et al., 2006). Specifically, it was shown that the initial opening of the BBB was related to the constitutive expression of MMP-2 and that TJPs may become loosened while remaining within the endothelial cell clefts. As the ischemic event progresses and an inflammatory response is induced, the expression of MMP-9 is increased along with other proteases and free radicals. This results in further degradation of tight junction proteins within the clefts of endothelial cell (Yang et al., 2006). Administration of tPA may contribute to the overall rate and extension of BBB breakdown by facilitating MMP activation (Montaner et al., 2003).

The temporal profile of gelatinolytic activity has been described in the hours to days after an ischemic event (Kelly et al., 2006; Rosell et al., 2006; Zhao et al.,

2006a) and Chapter 2 of this thesis describes the time-dependent activation in focal ischemia using tPA (Kelly et al., 2006). As MMP-2 is constitutively expressed and bound to the cell membrane by both a TIMP and an MT-MMP, in the early hours following an ischemic injury the expression levels of MMP-2 do not change (Rosell et al., 2006; Zhao et al., 2006a). MMP-9 is the gelatinase responsible for the early degradation of the BBB and MMP-2 does not become activated until 5-7 days later (Rosell et al., 2006; Zhao et al., 2006a). This longer latency to MMP-2 activation could represent an attempt at returning blood flow to the penumbral area through the process of angiogenesis. The differing temporal activities of the MMPs represents a fine balance in the proteolytic activity of MMPs. While early MMP activation may be detrimental by increasing the permeability of the BBB, later MMP activation may represent proliferation of cerebral blood vessels in the case of cerebral ischemia. Inhibition of MMPs is not only of interest in the case of cerebral ischemia, to lessen the extent of BBB breakdown, but also in cancer therapy development, to reduce the rate of angiogenesis, thereby limiting tumor growth.

MMP Inhibition

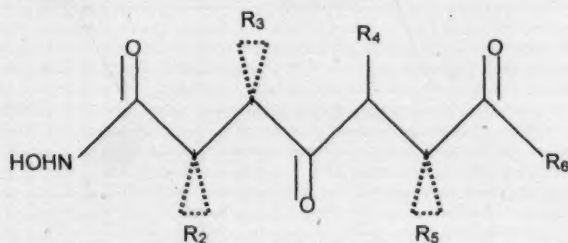
Pharmacological agents have been developed with the potential to downregulate MMP expression or activation however none has proven useful in the clinical setting (Cawston et al., 1993; Vincenti et al., 1994; Hanemaaijer et al., 1998; Crul et al., 2002). Physiological side effects, classified as a musculoskeletal syndrome are the primary reason that MMP inhibitors cannot be used clinically and it is also possible that there would be inhibition of the beneficial effects of MMPs (Gu et al., 2005). The benefits of reducing MMP expression and activation cannot,

however, be denied. Studies involving MMP-9 knock-out animals demonstrate a reduced lesion volume and reduced BBB breakdown after a transient cerebral ischemia (Campbell et al., 2004). However, it remains unclear whether the protection exerted by inhibition of MMP-9 is due to the inhibition of leukocyte recruitment at the level of the vasculature, or whether the MMP inhibition protects the brain from proteolytic activity.

The first generation of MMP inhibitors were primarily broad-spectrum inhibitors in that they recognized the overall structure of MMPs and were not designed to specifically inhibit a subclass of MMPs. CGS 27023A an early broad-spectrum MMP inhibitor studied in aortic atherosclerotic lesions, was not beneficial for treatment of the atherosclerotic lesion and it did reduce the extent of elastin destruction in the aortic media (Ferrans, 2002). Another approach to inhibition of MMPs is through the chelation of the zinc enzyme in the catalytic site. GM6001 has been widely used for this purpose as it binds the zinc to a hydroxamic acid group with high affinity (Ferrans, 2002). PD180557 and PD166793 are compounds that use carboxylic acid groups to chelate the zinc ion of MMPs. The problems with these compounds primarily involve their translation to humans. Hydroxamic acids are quickly metabolized by the liver and are not readily soluble in aqueous solutions (Ferrans, 2002). The zinc chelation properties of these compounds has also been questioned as they have such a strong affinity for zinc that they bind other metalloproteinases which are not MMPs (Overall and Kleifeld, 2006). It was this low specificity and systemic toxicity which led to the development of second generation MMP inhibitors however, treatment with these compounds exhibited

deleterious side effects involving the musculoskeletal system including joint pain, reduced mobility and stiffness (Overall and Kleifeld, 2006). Second generation MMP inhibitors were designed to chelate the MMP catalytic Zn^{2+} ion in a double-coordinating fashion, however, this made them unable to distinguish between the individual MMPs (Gu et al., 2005). Due to the potentially beneficial effects of MMPs, it would be ideal to develop an MMP inhibitor with high specificity. This has proven difficult due to the high structural homology of the MMPs and in particular the gelatinases (Emara and Cheung, 2006).

Figure 1.5. Structural formula of Hydroxamate.



BB-94, or batimastat, a synthetically derived, hydroxamic acid-based MMP inhibitor specifically mimics the site in the collagen substrate cleaved by the MMPs and works by competitive, reversible inhibition (Bjornsson et al., 2004). This was the first MMP inhibitor to enter clinical trials for cancer therapy (Rudek et al., 2002). This class of MMP inhibitors forms five-membered chelates and establishes two hydrogen bonds with the protein as it mimics an MMP substrate such as gelatin and

collagen (Rudek et al., 2002; Campestre et al., 2006). Not only does this MMP inhibitor have a broad-spectrum of inhibition, it is also relatively insoluble, has poor bioavailability when given orally, and is rapidly excreted (Bjornsson et al., 2004; Campestre et al., 2006). For these reasons it is only used as an investigative tool and has been dropped from clinical trials in cancer research (Rudek et al., 2002). Marimastat has recently been developed and extensively studied however, no consistent changes in MMP-2 and-9 downregulation have been noticed after administration (Rudek et al., 2002). Broad-spectrum MMP inhibitors are associated with musculoskeletal side effects along with administration and absorption problems, while more specific MMP inhibitors may interrupt the beneficial effects that MMPs can exert (Rudek et al., 2002). More recently, the tetracyclines have gained much attention for their MMP inhibitory properties. Tetracyclines mechanism of action for MMP inhibition is presumed to be chelation of the Zn^{2+} ion at the active site of the MMP (Rudek et al., 2002; Lalu et al., 2003). Along with its broad-spectrum MMP target, tetracyclines have the advantage of being readily absorbed, well-tolerated with minimal side effects and do not inhibit other classes of proteases (Golub et al., 1998; Rudek et al., 2002; Lalu et al., 2003).

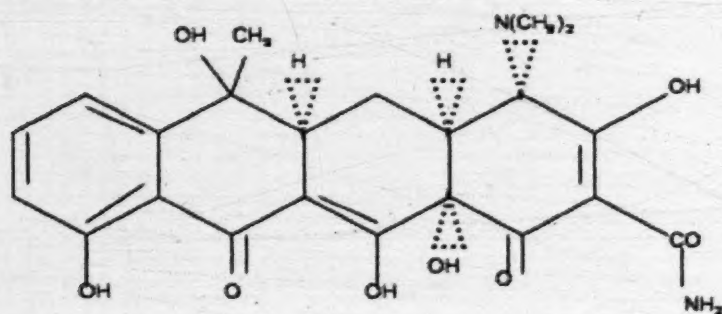
Tetracyclines

Tetracyclines have been used for many years for the treatment of various inflammatory diseases such as periodontal disease and gonorrhea (Golub et al.,

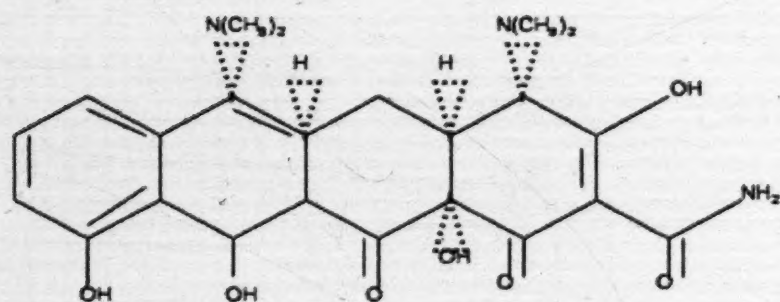
1998). The tetracyclines are bacteriostatic agents that have antimicrobial activity (Golub et al., 1998). The way in which tetracyclines act as antimicrobial agents is two-fold; by direct inhibition of protein synthesis, and indirectly through membrane perturbations that effect calcium movements in and out of the bacterial cell. The class of tetracyclines is inexpensive with a well-characterized safety profile. Minocycline (MINO) and doxycycline (DOXY) are second generation tetracycline derivatives that have gained interest in recent years as they cross readily into most bodily tissues including brain and cerebrospinal fluid (Fan et al., 2006; Meli et al., 2006). Both tetracycline derivatives have been shown in many models to be neuroprotective through their anti-inflammatory actions, free radical scavenging ability, inhibition of iNOS, and inhibition of specific caspases (Hanemaaijer et al., 1998; Fan et al., 2006; Lee et al., 2006a; Meli et al., 2006). Additionally, there has been recent interest in their use as inhibitors of MMP activation. A variety of mechanisms have been proposed for the tetracyclines inhibition of MMPs including: the inhibition of already-active MMPs; the inhibition of pro-MMP activation and the down-regulation of MMP expression (Golub et al., 1998; Ryan and Ashley, 1998). The original study investigating tetracyclines for MMP inhibition was conducted in 1983 and used minocycline to reduce excessive collagenase activity in the skin of diabetic rats (Ryan et al., 2001). The MMP inhibitory property of tetracyclines provided the possibility for a novel therapy for disease states characterized by breakdown of the extracellular matrix (Ryan et al., 2001). In one of the original studies by Golub et al., it was found that the tetracyclines were better able to inhibit collagenolytic activity than other antibiotics tested and that Ca^{2+} or Zn^{2+} inhibited

this function (Golub et al., 1998). This led to the discovery that the Ca^{2+} and Zn^{2+} binding site was responsible for the inhibiting the activation of MMPs, and more specifically, the gelatinases (Golub et al., 1983; Ramamurthy and Golub, 1983; Golub et al., 1998). It has been determined that the relative efficacy of tetracyclines and their chemically-modified counterparts, is correlated to their Zn^{2+} -binding capabilities (Golub et al., 1998; Hanemaaijer et al., 1998). A class of chemically-modified tetracyclines or CMTs, have been altered such that a dimethylamino group has been removed. The removal of the dimethylamino group results in a compound which retains its anti-MMP property while having lost its antimicrobial property (Golub et al., 1987; Golub et al., 1998; Hanemaaijer et al., 1998).

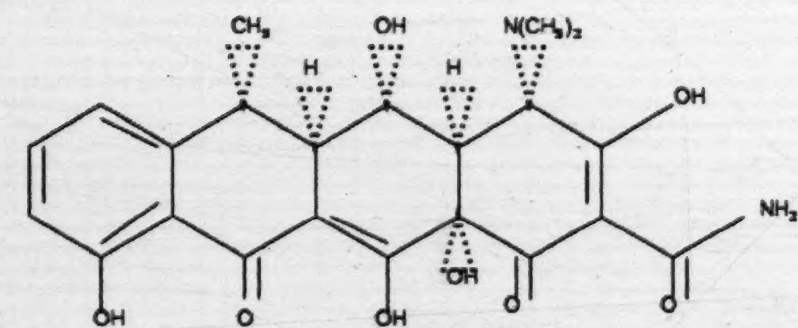
Figure 1.6. Structure of Tetracycline Derivatives. A. Structure of tetracycline. B. Structure of Minocycline. C. Structure of Doxycycline.



A.



B.



C.

DOXY, MINO and the aforementioned CMT were directly compared for their MMP inhibition properties, DOXY was found to be the most effective of the three compounds for the inhibition of MMPs (Ryan et al., 2001). DOXY has been shown to interact with the active MMP and inhibit the enzymatic activity by binding to the Zn^{2+} in the catalytic site (Sorsa et al., 1994). One study reported that DOXY binds to the pro-MMP altering the enzyme's conformation leading to excessive degradation of the pro-form and fragmentation of the molecule (Sorsa et al., 1994). However, this study has been riddled with controversy and has not been replicated since (Golub et al., 1998).

The ability of MINO and DOXY to inhibit MMPs extends beyond chelation of the Zn^{2+} ion; these two compounds can reduce human umbilical vascular endothelial cell proliferation (Fife et al., 2000; Yao et al., 2004), and DOXY has been shown to reduce smooth muscle cell migration by way of reducing MMP-2 and -9 activity in the arterial wall (Yao et al., 2004). DOXY inhibits MMP-2 and -9 and improves endothelial dysfunction (Camp et al., 2004). The potential therapeutic benefit of using tetracyclines and their derivatives extend quite far, they are able to interact with many downstream signaling pathways as well as interrupt the enzymatic activity of MMPs.

Downstream Signaling Effects of Tetracyclines

DOXY has been shown to not only reduce MMP activation but also to reduce the activation of $TNF\alpha$ and TACE, thus, the protective effect of DOXY can be partially attributed to its action as an anti-inflammatory agent. DOXY and MINO have been shown in many studies to inhibit microglial activation (Yrjanheikki et al.,

1999; Diguët et al., 2004; Jantzie et al., 2006). Microglia are the resident inflammatory cells of the CNS and they become activated following an ischemic event, during an inflammatory response and in various neurological disease states, such as multiple sclerosis, Alzheimer's disease and Parkinson's disease (Yrjanheikki et al., 1999; Diguët et al., 2004). Microglia can exert both trophic and toxic responses. Once activated they function by releasing a variety of cytotoxic substances such as reactive oxygen species, arachidonic acid metabolites and inflammatory cytokines such as TNF α and IL1- β (Yrjanheikki et al., 1999; Cho et al., 2000; Fan et al., 2006). Microglia can also be beneficial through the production of neurotrophic factors and growth factors (Lai and Todd, 2006). The activity of MMPs can also be upregulated by the same factors that are released by activated microglia, thus, inhibiting microglia can in turn, inhibit the activation of MMPs (Ferrans, 2002). The protective effects of MINO in neonatal hypoxic/ischemic injury have been attributed to its microglial inhibition and thus a reduction in oxidative/nitrosative stress (Fan et al., 2006). The reduction of inflammatory responses can also be extended to DOXY in studies involving atherosclerotic tissue and the stability of carotid plaques (Axisa et al., 2002). Here, DOXY was able to reduce MMP activation as well as interleukin levels and the vascular dysfunction associated with this increased inflammation (Lalu et al., 2006). This inhibition of the inflammatory response and nitric oxide synthase may act to reduce proteolytic activity (Axisa et al., 2002).

To date, MINO and DOXY have been shown to inhibit cerebral MMP-9 in a dose-dependent manner, at the mRNA and proteolytic activation level (Lee et al.,

2006a). There is evidence to show that MINO and DOXY mediate their respective MMP inhibition through different mechanisms. MINO mediates its MMP inhibitory action through an ERK and PI3K pathway, downregulating the transcription of MMP-2 and -9 (Lee et al., 2006a). It is as yet unknown through which downstream pathway DOXY is exerting its actions.

Angiogenesis – Involvement of tPA and DOXY

A common theme throughout this first chapter is the importance of the restoration of the cerebral microvascular circulation for functional recovery following an ischemic event (Zhang et al., 2000). For the endogenous regeneration of cerebral microvessels; an intact basement membrane on which endothelial cells can migrate and proliferate to reform vascular channels is required (Zhang et al., 2000). Exogenous administration of VEGF in the hour after focal ischemia has been shown to increase BBB permeability and increasing hemorrhagic transformation, if administered 48 hours after focal ischemia, there is an improvement in neurological recovery (Zhang et al., 2000; Valable et al., 2005). MMP activation, ECM breakdown and the initiation of endothelial cell proliferation and migration are all intricately linked in the balance between BBB breakdown causing hemorrhage and the BBB breakdown which is required for the formation of new cerebral blood vessels to restore blood flow to an ischemic area.

The “angiogenic-switch” refers to the transition from a prevascular phase to a vascular phase, in which endothelial cells are proliferating and migrating (Pepper,

1997; Lamoreaux et al., 1998). As endothelial cells migrate, they form endothelial sprouts and eventually form a new capillary network (Lamoreaux et al., 1998). VEGF functions as a potent angiogenesis-inducing agent by way of inducing increased permeability in the ECM, acting as a chemoattractant for endothelial cells, and encouraging the proliferation of endothelial cells (Lamoreaux et al., 1998; Brekken and Thorpe, 2001; Kaya et al., 2005). The VEGF molecule exists as a dimer and binds to one of two receptors, expressed on endothelial cells (Brekken and Thorpe, 2001; Kaya et al., 2005). For the induction of angiogenesis, an angiogenic stimulus, such as hypoxia, initiates endothelial cell activation including cell proliferation and proteolysis of the underlying basement membrane matrix (Iruela-Arispe et al., 1991; Lamoreaux et al., 1998; Haas and Madri, 1999). MMP-9 is the functional component of this angiogenic switch as it mobilizes VEGF from an extracellular reservoir (Lamoreaux et al., 1998). The link between VEGF and MMPs is further strengthened by the observation that addition of VEGF to cultured endothelial cells, causes the reduction of TIMP-1 in conditioned media (Lamoreaux et al., 1998), this would enable MMP-9 to assume its proteolytic function. It is difficult to say what process cerebral endothelial cells will undergo to manipulate endothelial cell homeostasis as it has been found that the microenvironment for endothelial cells of different origins may produce differing enzymes when stimulated to proliferate and migrate. For example, macrovascular endothelial cells have been shown to secrete collagen type III while microvascular endothelial cells secrete collagens type I and IV (Iruela-Arispe et al., 1991; Lamoreaux et al., 1998). Following a myocardial ischemia, MMPs are activated but in a pro-angiogenic

capacity thereby promoting endothelial cell migration (Bergers et al., 2000). The literature does not agree on the involvement of one specific MMP in the initiation or progression of angiogenesis, as with the matrix surrounding endothelial cells, it appears that the mitogenic factors regulating angiogenesis are reliant on the microenvironment.

With regards to the inhibition of MMP activity, there is some controversy in the literature regarding the anti-angiogenic effects of the tetracyclines. The tetracyclines have been found to be neuroprotective, by reducing infarct size in more than one model of focal ischemia, and the tetracyclines have also been found to encourage the migration and proliferation of new neurons from the subventricular zone (Sapadin and Fleischmajer, 2006). Within the subventricular zone, an area known for its neural precursor cells, neuronal and endothelial precursors proliferate together, and this provides a vascular niche for neurogenesis (Cleaver and Melton, 2003). While in studies of tumor progression DOXY has been shown to reduce angiogenesis by inhibiting VEGF (Fife et al., 2000). Thus it may not be the inhibition of MMPs which suppresses new blood vessel formation. MMP-2 deficient mice only demonstrate partially-suppressed tumor growth and in mice deficient in MMP-9, reduction of vessel formation is limited to long bones and may be due to an inability of growth factors to be released from the matrix rather than a loss of the migratory abilities of endothelial cells (Haas and Madri, 1999).

In studies of FCI, development of neuroprotective strategies focusing on the arrest of the apoptotic cascade have not yet proven clinically successful. The focus of this thesis is on the restoration of blood flow to the ischemic region through the

thrombolytic action of tPA then examining the downstream effects on the BBB that tPA has. The destructive capabilities of tPA on the BBB have been thought to be due to the activation of MMPs. Recent studies in the literature have focused on the inhibition of MMPs though none has examined this in combination with tPA thrombolysis.

Overall Objective

The studies described herein were designed to demonstrate the mechanisms underlying the evolution of BBB breakdown toward potential hemorrhagic transformation which can occur following tPA administration for the treatment of acute stroke. Under investigation were the acute temporal profile of MMP activation, within 24 hours of ischemic induction and tPA thrombolysis. Following this, I examined MMP inhibition with DOXY in combination with tPA thrombolysis in the same model of focal ischemia seven days after the insult. Use of DOXY had never been examined in combination with tPA thrombolysis, for the treatment of focal cerebral ischemia. The interesting results from this study led to the examination of the downstream molecular events following tPA and DOXY administration. To investigate this, an adult cerebral endothelial cell culture was used with the application of pharmacological inhibitors of known downstream effectors. This allowed the outline of the deleterious interaction between MMP inhibition and tPA thrombolysis that observed *in vivo*. Despite the prevalence of

stroke among an aged population, the MMP activation induced by tPA has never been investigated in an aged model of cerebral ischemia. Using rats older than one year, tPA thrombolysis and subsequent MMP activation was examined within an aged vasculature. This final study also investigated the changes in TJP, MMP and laminin expression with age in the naïve rat.

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Chapter 2:
Matrix metalloproteinase activation and blood-brain
barrier breakdown following thrombolysis

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Introduction:

Thrombolysis with tissue plasminogen activator (tPA) is the only pharmacological treatment option currently available for the treatment of ischemic stroke. When administered intravenously within 3 hours of symptom onset, tPA has been shown to be beneficial (Pfefferkorn and Rosenberg, 2003), albeit to a small population of patients. However, reperfusion with tPA has been associated with devastating sequelae including, an increased risk of hemorrhagic transformation and edema (Vivien and Buisson, 2000; Yepes et al., 2003). Though the molecular mechanisms underlying this transformation have yet to be fully elucidated, hemorrhagic conversion occurs as a result of the destruction of the blood-brain barrier (BBB) and an increase in vascular permeability (Yepes et al., 2003). Matrix metalloproteinases (MMPs), a family of zinc-dependent proteases, have recently been implicated in the reperfusion injury that follows cerebral ischemia (Sumii and Lo, 2002; Wang et al., 2003b). Activation of these MMPs is thought to be partially responsible for the breakdown of the BBB, due to their ability to degrade components of the extracellular matrix (Romanic et al., 1998; Yepes et al., 2003; Tsuji et al., 2005). It is possible that tPA activates MMPs through the plasminogen-plasmin system, thereby weakening blood vessels and predisposing them to rupture (Sumii and Lo, 2002; Pfefferkorn and Rosenberg, 2003; Tsuji et al., 2005). Matrix metalloproteinase-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) are the focus of many studies of cerebral ischemia because of their substrate specificity for fibronectin, laminin and collagen type IV, structural components of the BBB

(Heo et al., 2005). To date, no study has examined the temporal profile of MMP activation and BBB breakdown following a tPA-treated focal ischemic event.

The objective of this project was to determine the time course of activation of both MMP-2 and MMP-9 as well as the BBB breakdown within the first 24 hours following the administration of tPA in a rat model of focal embolic cerebral ischemia. The embolic stroke model used here is well established in stroke research and more closely mimics the human stroke than other models of cerebral ischemia (Zhang et al., 1998; Krueger and Busch, 2002). The model uses thrombin mixed with arterial blood drawn from the middle cerebral artery to create a reproducible and predictable thromboembolic occlusion.

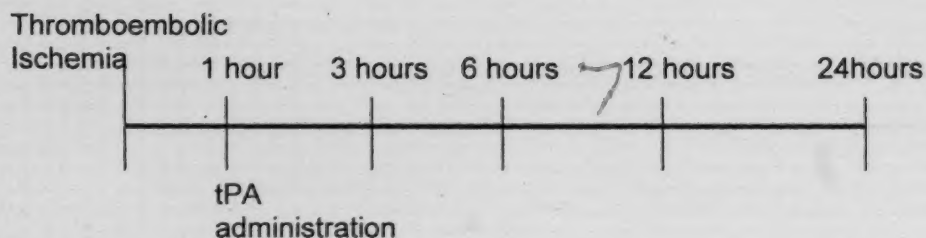
Our results demonstrate an increased activation of MMP-9 in tPA-treated animals 6, 12, and 24 hours following ischemic injury. The activation of MMP-2 remained unchanged throughout all time points studied, although when compared to saline treatment, tPA administration increased MMP-2 activity. The destruction of structural proteins of the BBB, namely, laminin and fibronectin, closely follows in time, the activation of MMP-9. Taken together, our results suggest that early inhibition of MMP-9 could reduce the amount of BBB damage and thereby the hemorrhagic transformation conferred by tPA administration.

Materials and methods:

Male Sprague-Dawley rats (locally obtained, 250-300g) were housed in a humidity and temperature-controlled environment with a 12-hour light/dark cycle. Care and use of animals complied with guidelines as outlined by the Canadian Council for Animal Care.

Rectal temperature was maintained at 37°C with a thermostat-controlled heating pad (Harvard Apparatus; Holliston Massachusetts, USA), during surgical procedures. Animals were randomly assigned to one of four experimental groups: Ischemia-Saline; Ischemia-tPA; Sham-Saline; or Sham-tPA. Each group included all four time points of interest: 3, 6, 12 and 24 hours following induction of ischemia or sham operation. Total N=120; group n=6.

Figure 2.1. Schematic of experimental procedure.



Focal Cerebral Ischemia

Animals were anesthetized using inhalant halothane, (3% to induce and 1.5% for maintenance) in 70% oxygen and 30% nitrogen. For this study we used a clinically relevant thromboembolic model of focal cerebral ischemia (Wang et al., 2001; Kelly et al., 2006). Briefly, a surgical incision was made in the ventral neck to expose the common, internal and external carotid arteries. The external carotid artery (ECA) was fully ligated for insertion of a PE-10 catheter (Fisher Scientific, New Jersey, USA). The catheter was advanced through the internal carotid artery (ICA) to the distal opening of the middle cerebral artery (MCA). Blood was drawn from this region and mixed with thrombin contained in the catheter to form a clot. Clot formation was allowed to progress for 15 minutes and the clot was deposited followed by a small saline injection advancing the clot to the MCA. The catheter was withdrawn after 15 minutes, animals were then returned to their cages for recovery. Those animals randomly assigned to a sham group received the same surgical procedure with the exception of clot formation and deposition. Animals were monitored continuously throughout the recovery period.

One hour following sham or ischemic operations, animals received tail vein injection of saline or tPA (10 mg/kg; Genentech, San Francisco, California, USA). The administration of tPA was in conjugation with clinical guidelines, human recombinant tPA (Actilyse) is 10 times less potent at activating rodent plasminogen than it is at activating human plasminogen (Lijnen et al., 1994; Benchenane et al., 2004), therefore we use a dose 10 times more concentrated (10mg/kg).

Three, six, twelve or twenty-four hours following the surgery, animals were euthanized and brains quickly removed and blocked for analyses of infarct volume, breakdown of the BBB components, and regional activation of MMP-2 and -9.

Measurement of Infarct Volume

One 2-mm coronal section of brain in the known infarct zone (Bregma + 0.20 mm according to The Rat Brain Atlas (Paxinos and Watson, 1998) as incubated in 2, 3, 5-triphenyltetrazolium chloride (TTC) at room temperature. The same representative 2-mm section was obtained from each brain studied. The size of the infarct remained consistent with little variability within groups observed. Stained tissue sections were scanned using HP ScanJet software and converted to a jpeg file in Adobe Photoshop. The volume of infarcted tissue was quantified using a standard image analysis technique where the freehand tool was used to trace the infarcted area measured in pixel size (Krueger and Busch, 2002; Tureyen et al., 2004). The possibility of edema enlarging the infarcted tissue is taken into account by measuring the area of the surviving tissue on the ipsilateral side and subtracting it from the area on the contralateral side (Swanson et al., 1990). TTC staining as a measure of infarct volume is commonly used and is equivalent to the cresyl violet or hematoxylin-eosin staining methods for quantifying infarct volume following focal cerebral ischemia (Bederson et al., 1986).

Gelatin Zymography

Remaining brain tissue containing the infarct zone was immediately flash-frozen in ice-cold isopentane and stored at -80°C until used for further histological analyses. Specifically, one set of brains (n=3) from each of the four groups and each

of the four time points were used for gelatin zymography (total N=48). Another set of brains (n=3, from all groups and time points examined; total N=48) was used for cryosectioning and immunostaining. Tissue used for gelatin zymography was divided into ipsilateral ischemic hemispheres and contralateral non-ischemic hemispheres and further dissected into striatal and cortical regions. This dissection process was performed to thoroughly compare MMP activation throughout both the core infarct area (striatum) and the penumbral infarct area (cortex) over time. Brain samples were homogenized in lysis buffer containing 50mM Tris-HCl, 320mM sucrose, 1mM dithiothreitol, 10 μ g/mL leupeptin, 10 μ g/mL soybean trypsin inhibitor and 2 μ g/mL aprotinin. After centrifugation at 9000 rpm for 20 mins, supernatant was collected and total protein concentration was determined by Bradford's protocol. Equal amounts (40 μ g/mL) of total protein extracts were prepared by mixing with lysis buffer and a loading reagent. Prepared samples (2 μ g/ μ L) were loaded and separated by 8% acrylamide gel containing 0.1% gelatin as a substrate. Purified human MMP standards (Oncogene) were loaded in one lane at a final protein concentration of 10 μ g/100 μ L. Upon separation by electrophoresis, gels were incubated in renaturing buffer (2.5% Triton solution) for 1 hour. Gels were then incubated in incubation buffer (50mM Tris-HCl, 0.15M NaCl, 5mM CaCl₂) at 37°C until bands appeared. Gels were stained in Coomassie blue R-250 for one hour and destained accordingly. MMP activation appeared as transparent bands on a blue background. Images of gels were captured by scanning each gel on an HP ScanJet flatbed scanner; bands were quantified by densitometry using ImagePro Express Analysis system.

IgG Extravasation

This technique is used as a gross measure of BBB breakdown (Calingasan et al., 1995; Todd and Butterworth, 1999). By incubating slides with species-specific IgG, one can microscopically visualize and quantify the extent of leaky blood vessels as the IgG contained within vessels leaks into the parenchyma. Frozen coronal sections (20 μ m) were obtained from brain tissue not incubated with TTC (n=3, each time point). Sections were fixed in formalin and dehydrated through a series of graded alcohols and xylene. Sections were then incubated for 1 hour at room temperature with anti-rat IgG (1:250 in PBS). Peroxidase activity was visualized with 3,3'-diaminobenzidine (DAB) and hydrogen peroxide in PBS. Blood vessel leakiness was quantified by field scoring areas known to be in the infarcted area.

Immunohistochemistry

Immunohistochemistry was performed to assess the cellular localization of MMP-2 and -9, and to determine extent and location of the degradation of both laminin and fibronectin. Vascular integrity was measured by a loss of positive staining for these two structural components of the BBB. This was performed for all groups at all time points under study. Frozen coronal sections (20 μ m) were obtained from brain tissue that was not incubated with TTC (n = 3, from each time point). Sections were fixed in formalin (except when antibodies recognizing MMP-2 and -9 were used) and were dehydrated through a series of graded alcohols and xylene. Quenching of endogenous peroxidase activity was achieved through incubation in 50% methanol containing 0.03% hydrogen peroxide. Non-specific binding was blocked with a universal blocking solution and 0.2% triton-X. Sections were then incubated

overnight with primary antibodies, these included: rabbit anti-laminin (1:100); rabbit anti-fibronectin (1:600); rabbit anti-MMP2 (1:50); mouse anti-MMP9 (1:100); rabbit or mouse anti-glial fibrillary acidic protein (GFAP) (1:1000); and mouse anti-NeuN (1:250). Sections were washed with PBS and incubated with appropriate secondary antibodies (1:200). Those secondary antibodies not conjugated to fluorescent chromogens were incubated for 30 min with an avidin-biotin complex and visualized by incubation with DAB and hydrogen peroxidase. Negative controls were subject to all treatment conditions with the exception of primary antibody incubation. Loss of laminin and fibronectin was quantified by counting positive staining in striatum and cortical regions of both ipsilateral and contralateral hemispheres.

In situ zymography

This technique was used to localize net gelatinolytic activity. FITC-labeled DQ gelatin is used as a substrate for gelatinases. Cleavage of gelatin substrate by gelatinases results in increased intensity of fluorescence through release of the quenched fluorochrome. Tissue sections were obtained as described above. Thawed sections were incubated for 24 h in reaction buffer (0.05 M Tris-HCl; 0.15 M NaCl; 5 mM CaCl₂; 0.2 mM NaN₃; pH 7.6), containing 40 µg/mL DQ gelatin. Sections were then fixed in formalin and coverslipped with gel mount. Slides used as negative controls were incubated with reaction buffer not containing DQ gelatin. Fluorescence was visualized under fluorescence microscopy and photographs obtained with CoolSnap Pro digital camera and Image Pro Plus Software.

Statistical analyses

Quantitative data were expressed as mean + SEM. Statistical comparisons for infarct

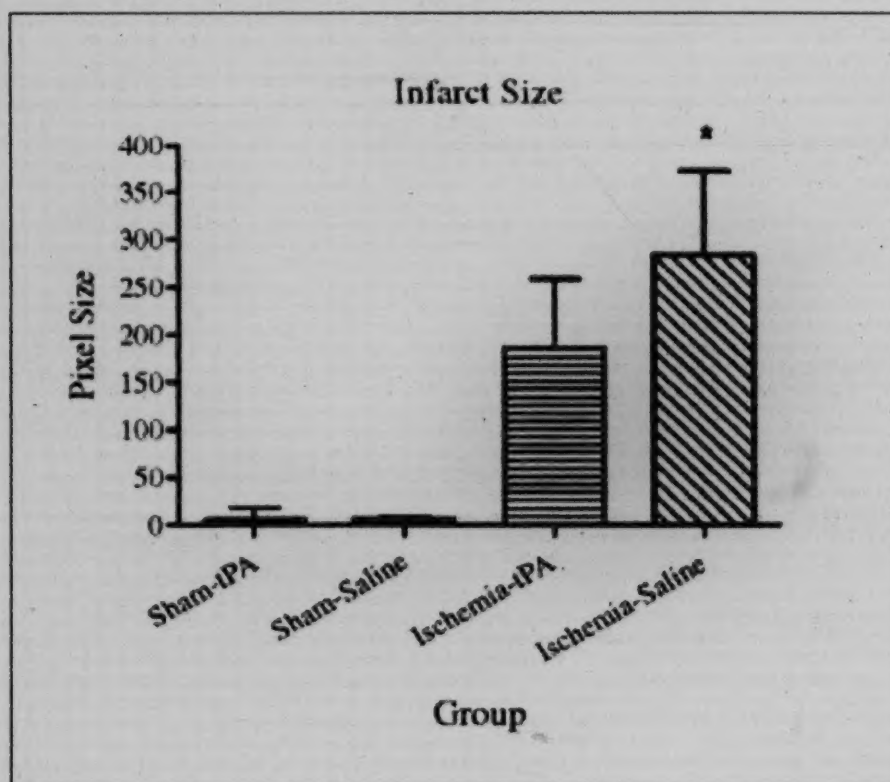
size were done using two-way ANOVA followed by Bonferroni post hoc analyses. Comparisons for laminin and fibronectin staining were performed using nonparametric Kruskal–Wallis test and Bonferroni post-hoc analysis. Differences with $P < 0.05$ were considered statistically significant.

Results:

Infarct volume

Cerebral infarct was measured by TTC staining in the area known to be the most damaged following occlusion of the middle cerebral artery. Figure 2.2 shows a histogram of overall infarct volume in the four treatment groups, as well as a sample section obtained from this study. Overall infarct volumes were Sham-tPA: 5.80 ± 4.55 [mean $\pm$ SE]; Sham-Saline: 5.00 ± 4.23 ; Ischemia-tPA: 186.1 ± 73.45 ; Ischemia-Saline: 284.8 ± 88.74 (all $P < 0.05$) tPA significantly reduced infarct volume in ischemic animals. Although these data are widely published, it is included here to demonstrate the validity of our model (Vivien and Buisson, 2000; Gautier et al., 2003a; Graham, 2003; Yepes et al., 2003).

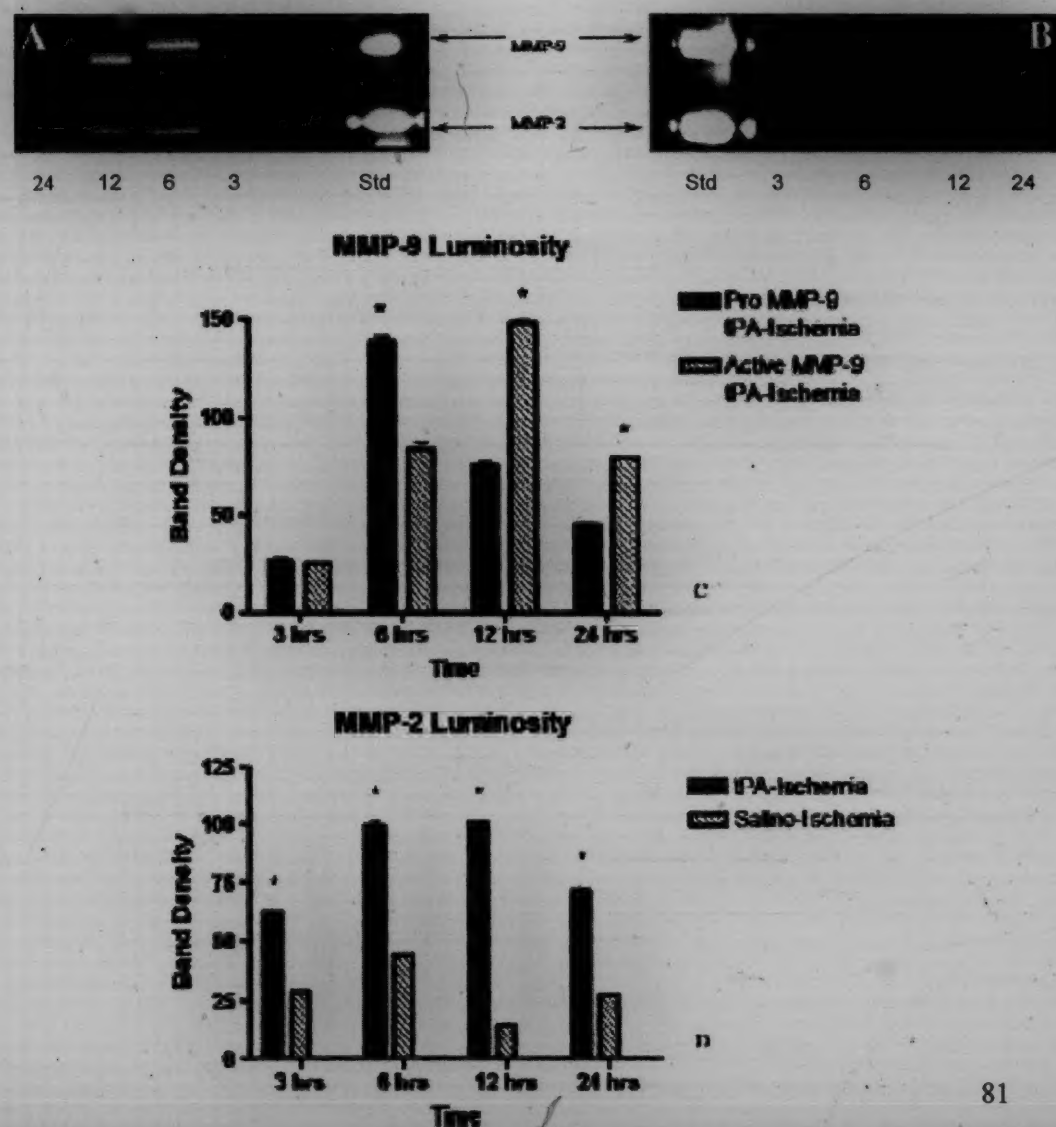
Figure 2.2. Infarct Size. Infarct volume measured by TTC staining. tPA treatment significantly reduces infarct volume compared to saline treatment.



Gelatin zymography

Results obtained from gelatin zymography experiments demonstrate an increase in MMP-9 activation at 6, 12, and 24 h following ischemia in tPA-treated animals. The pro-form of MMP-9 was increased significantly at 6 h only. These data are represented in Fig. 2.3 A. The contralateral (non-ischemic) hemisphere of tPA-treated animals did not demonstrate any changes in either the pro- or active forms of MMP-9 at any of the time points examined (data not shown). Similarly, saline treated ischemic animals and sham-operated animals did not demonstrate MMP-9 activation (Fig. 2.3 B). The pro-form of MMP-9 observed in the homogenates from these groups was used as a baseline measure for any changes that occurred. These results were obtained in both the cortex and striatum of all animals. In contrast to the results obtained from MMP-9, a band depicting MMP-2 activation was observed at all time points in both treated and non-treated animals. This result was consistently obtained in both sham-operated and ischemic groups. Although this band appeared in all groups studied at all time points examined, there were no significant temporal changes observed in the activation of MMP-2. This result of MMP-2 being constitutively present is expected based upon the literature (Gasche et al., 1999; Asahi et al., 2000; Gasche et al., 2001; Aoki et al., 2002; Heo et al., 2005). However, a significant difference in the activity of MMP-2 between saline-treated animals and the tPA-treated animals was observed. This significant difference was again only observed in ischemic groups.

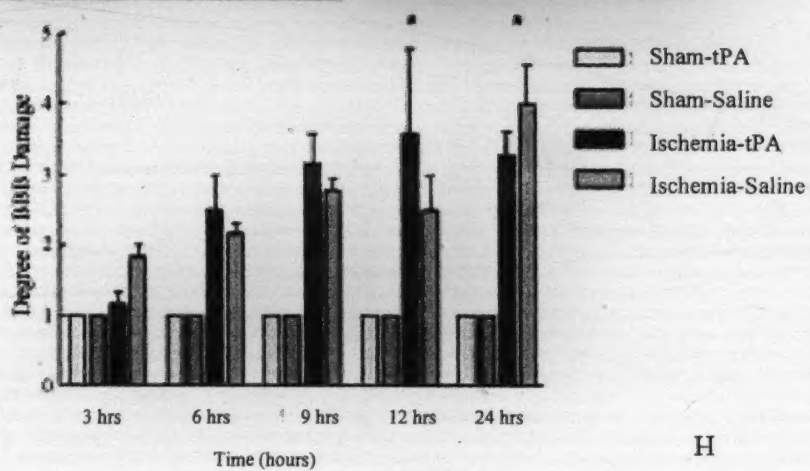
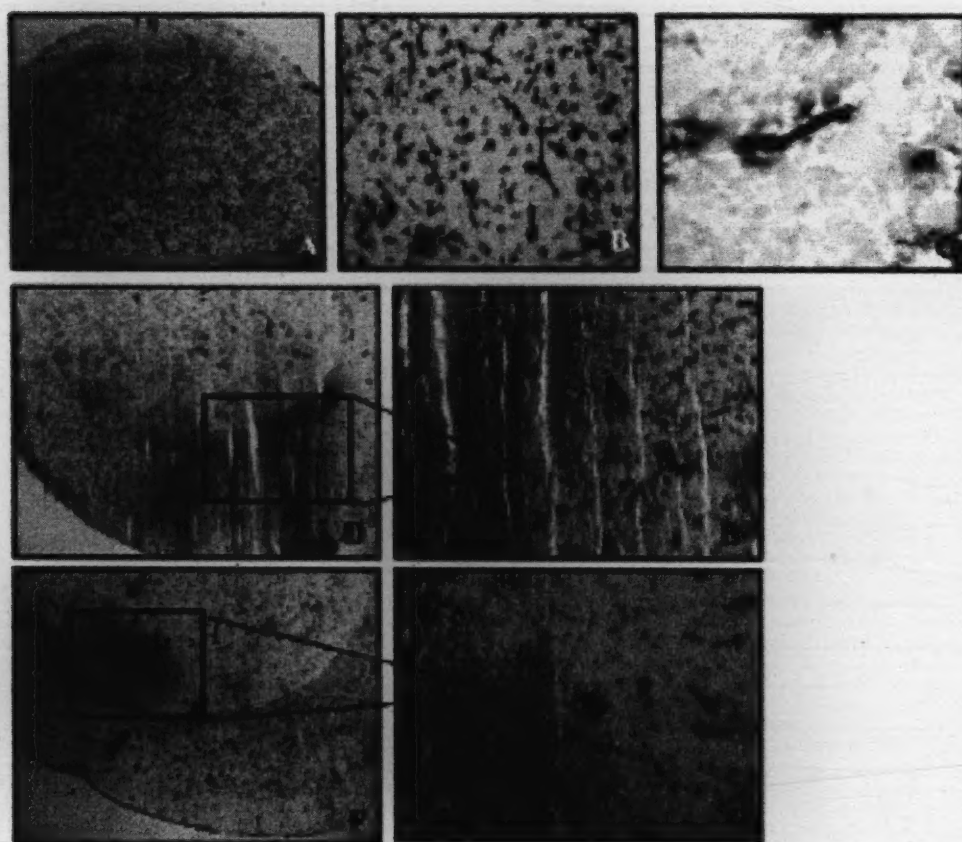
Figure 2.3. Representative zymograms. A. Zymogram shows gelatinolytic activity of tPA-treated ischemic animals at all time points. MMP-9 activity observed at 6, 12, and 24 hours after ischemia, and increased pro MMP-9 only at 6 hours following injury. MMP-2 activation is the same at all time points. B. Zymogram depicts gelatinolytic activity of saline-treated sham animals at all time points. Here, MMP-2 activity at all time points with complete absence of MMP-9 activity. C. Histogram represents band luminosity at all time points studied for MMP-9. Asterisks represent significant differences when active MMP-9 is compared to pro-MMP-9. Stds – standards of human MMP-2 and -9. D. Histogram represents band luminosity for MMP-2 at all time points measured. There were no significant differences when MMP-2 was examined over time, but activation levels differed significantly between tPA-treated animals and saline-treated animals, asterisks depict the significant results.



IgG extravasation

This gross measure of BBB breakdown demonstrated that non-treated animals showed IgG extravasation at all time points, with 24 h exhibiting the greatest IgG leakage from blood vessels (Figs. 2.4 D and E). In tPA-treated animals, 12 h appeared to be the threshold for BBB integrity. Beyond this time point, blood vessels became significantly more permeable. Sham-operated animals demonstrated no leeching of IgG from cerebral blood vessels (Figs. 2.4 A–C). Fig. 2.4 H displays a histogram of the relative IgG staining in the parenchyma.

Figure 2.4. IgG Extravasation. A-C. Represent a sham-saline-treated animal. Blood vessels are fully intact and IgG can be seen within them, especially in C. D-E. Represent a saline-treated ischemic animal 24 hours after ischemia. IgG has leaked into the brain parenchyma, and there is a clear border zone between leaky vessels and those that are intact. F-G. Represent a tPA-treated ischemic animal after 6 hours of ischemia. Leakage from vessels at this time point is more punctate and distinct. A,D,F. Magnification 4X. B,E,F. Magnification 10X. C. Magnification 20X. H. Histogram depicting the extravasation of IgG. Saline-treated ischemic animals demonstrate BBB breakdown as early as 3 hours. tPA-treated ischemic animals demonstrate IgG extravasation after 6 hours of ischemia.

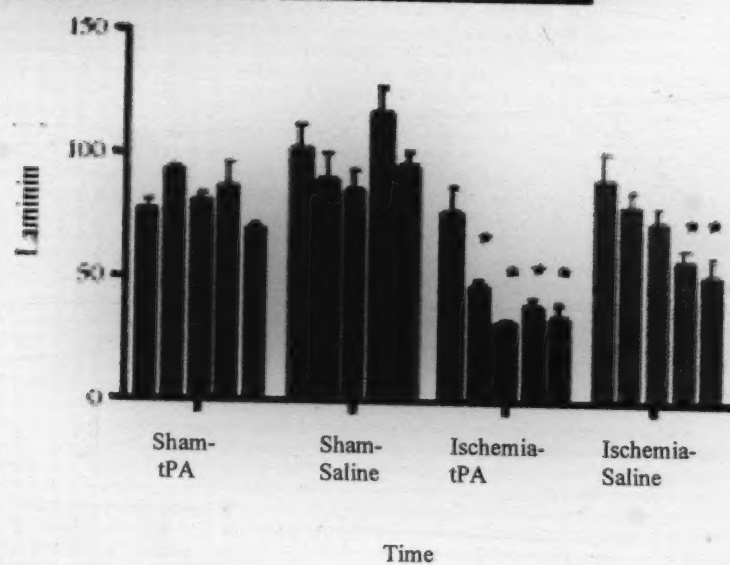
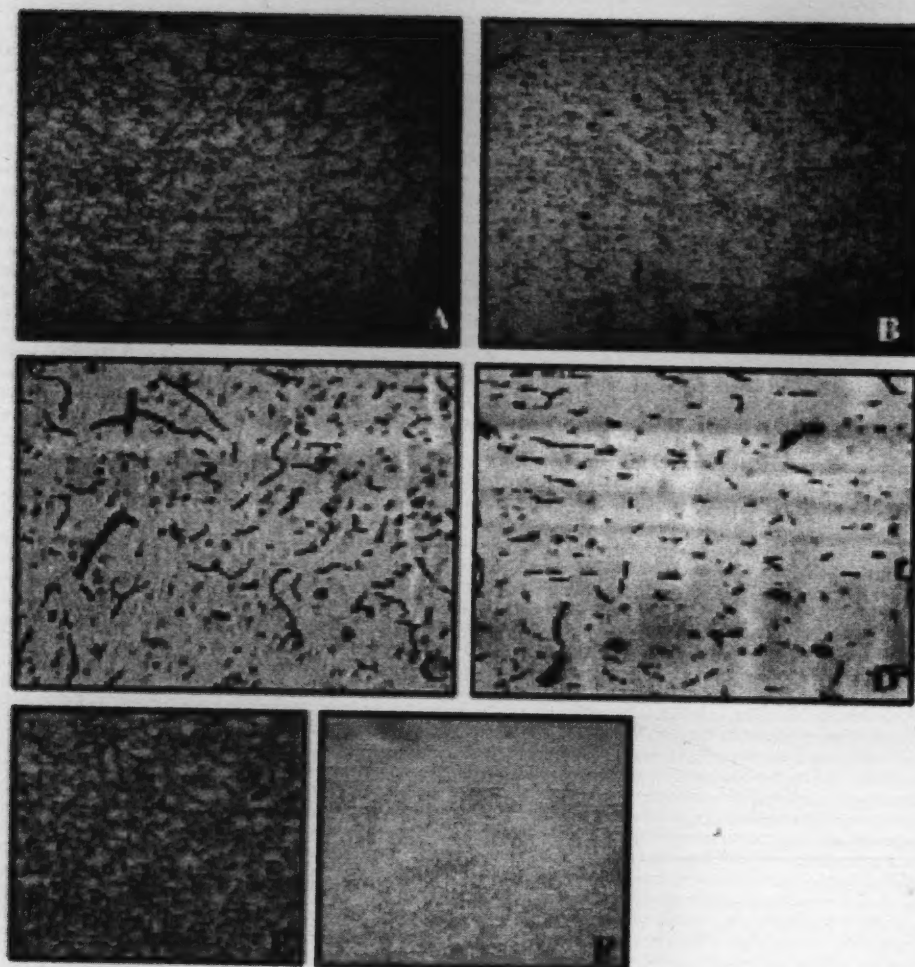


H

Immunohistochemistry

Immunostaining was performed on frozen tissue sections to measure the extent of breakdown of structural components of the BBB and to localize MMP-2 and -9 to specific cell type. Laminin and fibronectin were examined as they are substrates for the proteolytic activity of MMP-2 and -9 and are key structural components of the BBB. The analyses of immunoreacted sections from all groups revealed that laminin breakdown occurred within 6 h following ischemia in both treated and non-treated animals. The maximal amount of laminin breakdown occurred at 9 h following ischemia in treated animals and at 24 h non-treated animals. These data are shown in Fig. 2.5. Note the difference in the amount of laminin staining between contralateral and ipsilateral hemispheres. The ipsilateral or ischemic hemisphere demonstrates significantly less laminin immunostaining than the contralateral hemisphere. Also of note, is the morphology of the blood vessels which are present in either hemisphere. In ischemic areas, vessels have a non-continuous appearance suggesting that they have begun to lose their integrity when compared to the non-ischemic hemisphere.

Figure 2.5. Immunostaining for the structural protein laminin. A-B. Sections obtained from the same tPA-treated ischemic animal, 6 hours post-ischemia. A. Represents the left, contralateral hemisphere. B. Represents the right, ipsilateral ischemic hemisphere. Note the vast difference in the presence of laminin in each section. C-D are from the same saline-treated ischemic animal after 24 hours. C. Represents the left, contralateral hemisphere. D. Represents right, ipsilateral hemisphere. Again, note lack of laminin staining in panel D. Also of note is the broken appearance of blood vessels present. E. Immunostaining for laminin in a tPA-treated sham animal. Note all blood vessels appear intact. This is representative of all tPA-treated sham animals at all time points. F. Negative control for laminin immunostaining. Magnification: 10X. G. Histogram depicting loss of laminin immunostaining over time. Each group contains a bar representing a time point in increasing order. All ischemic animals demonstrate loss of the structural protein laminin after 6 hours. In tPA-treated ischemic animals, the loss of laminin is significant compared to both sham groups at 6, 12 and 24 hours and significantly different from non-treated ischemic animals at 6 hours. Ischemic animals treated with tPA showed more laminin loss than those animals that received saline following ischemia at the 6 and 9 hour time points.

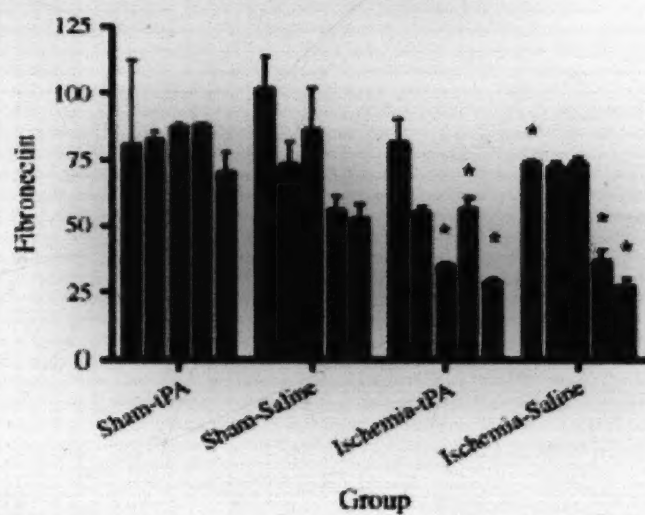
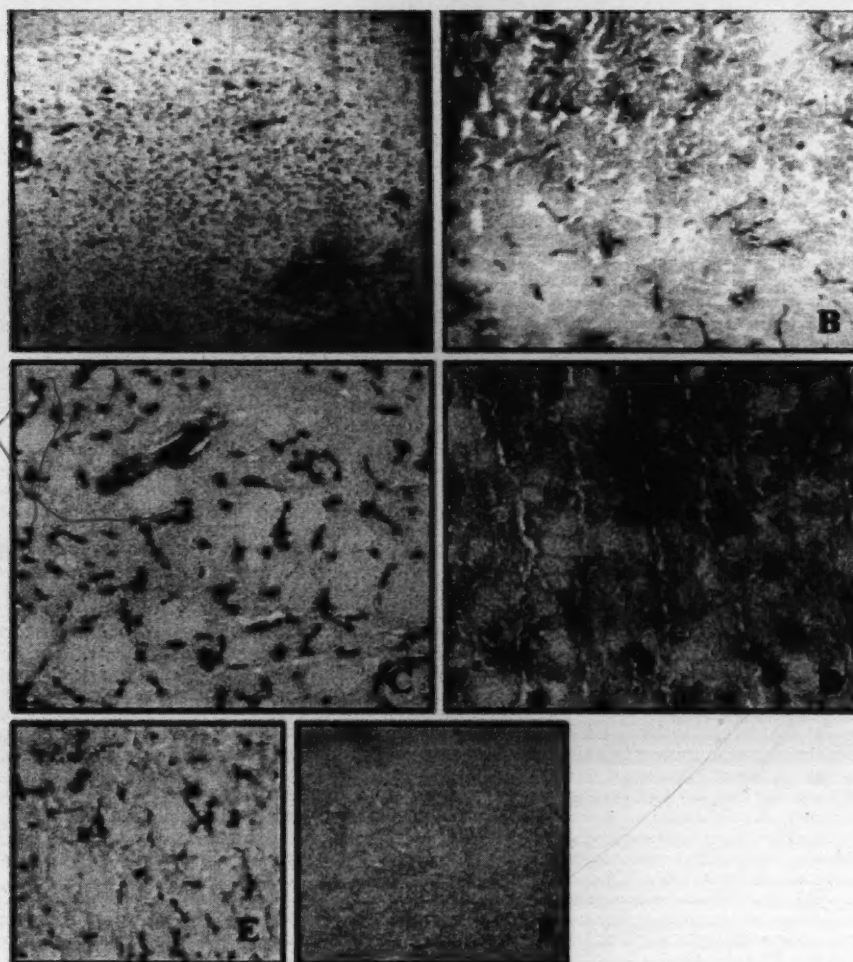


G

Fibronectin breakdown followed a similar time course as laminin breakdown in tPA-treated animals. Both laminin and fibronectin destruction were restricted to the ischemic hemisphere. Fig. 2.6 depicts fibronectin destruction after 9 h of ischemia in a tPA-treated animal. Non-treated animals did not demonstrate any significant destruction of fibronectin until 12 h following ischemia whereas sham-operated animals did not show any breakdown of fibronectin (Figs. 2.6 E and F).

Figure 2.6. Immunostaining for the structural protein fibronectin. A-B.

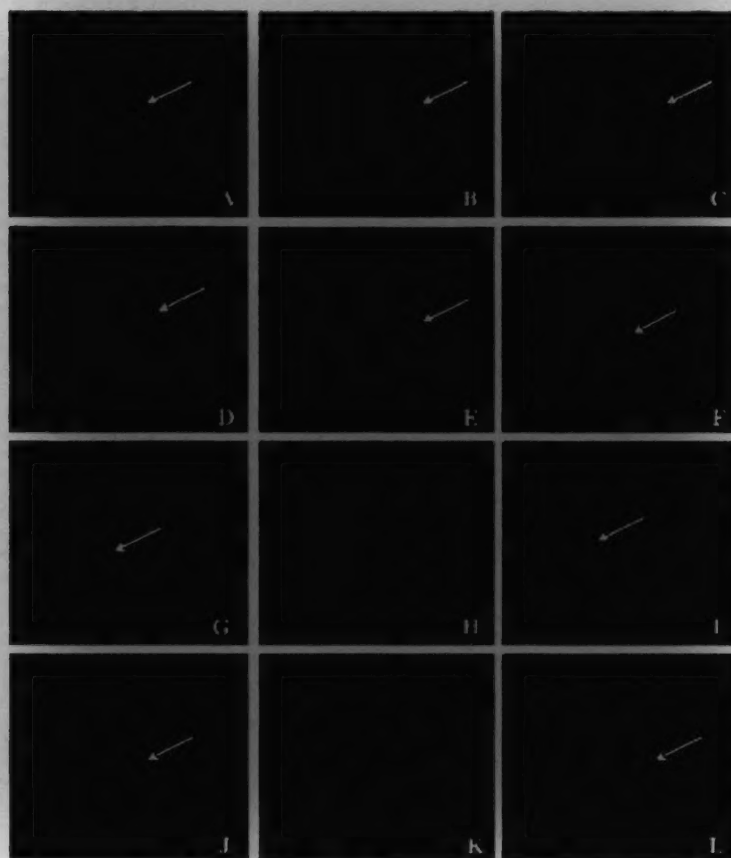
Figures are representative of a tPA-treated animal after 9 hours of ischemia. Note the breakdown of fibronectin which resembles the leakiness of IgG in previous figures. A. Magnification 4X. B. Magnification 10X. C-D. Sections are from the same animal saline-treated, ischemic animal at 24 hours. C. Photomicrograph representative of the left, contralateral hemisphere. D. Represents the right, ipsilateral hemisphere. Note the different patterns of staining between the two hemispheres. E. Represents a sham, saline-treated animal. Fibronectin surrounding blood vessels is intact and there is no leakage to the surrounding parenchyma. F. Negative control for fibronectin immunostaining. G. Histogram depicting breakdown of fibronectin over time. tPA-treated ischemic animals demonstrate loss of fibronectin after only 6 hours of ischemia, while non-treated animals compared to sham animals at 12 and 24 hours following ischemia. The loss of fibronectin is significant for tPA-treated ischemic animals compared to sham-animals at 3, 12, and 24 hours after ischemic injury.



Localization of MMPs through immunohistochemistry showed that MMP-2 was largely but not solely, restricted to neuronal cells throughout the brain (Figs. 2.7 A–C). This expression of MMP-2 did not change based on treatment or time point studied. MMP-2 was also observed to be present in astrocytes throughout both hemispheres in non-treated animals only, including both sham and ischemic animals. The glial expression of MMP-2 was rarely observed only in tPA-treated animals (Figs. 2.7 D–F). This result is in concordance with a study examining MMP inhibition in superoxide-dismutase knockout mice (Gasche et al., 2001).

Immunostaining demonstrated that MMP-9 was not expressed in either neuronal or glial cells (Figs. 2.7 G–L). This was consistent across all treatment conditions and time points. MMP-9 immunostaining was largely detected extracellularly. This extracellular expression of MMP-9 was expected since the enzyme acts extracellularly to degrade the proteins of the extracellular matrix thereby compromising the BBB.

Figure 2.7. Double immunofluorescent staining. A. Depicts NeuN staining, for neurons. B. Depicts immunostaining for MMP-2. C. The merged image. This section is from a tPA-treated ischemic animal, white arrows indicate a neuronal cell body expressing MMP-2. MMP-2 localized to neuronal cells is consistent among all animals included in the study. D. Depicts GFAP staining, marker for astrocytes. E. Depicts immunostaining for MMP-9. I. Overlaid image demonstrating no MMP-9 expression in astrocytes (white arrows in panels G and I). Sections are from non-treated ischemic animals. (G,H,I). J-L are obtained from tPA-treated ischemic animals. J. Immunostaining for GFAP. K. Immunostaining for MMP-9. L. Overlaid image, showing a lack of MMP-9 expression in astrocytes (white arrows).

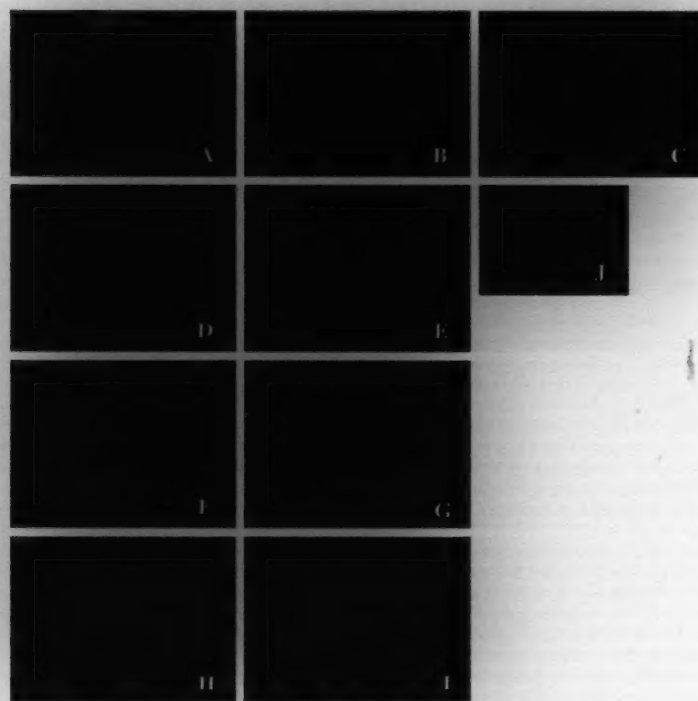


In situ zymography

The gelatin zymography described above does not accurately regionalize the activity of MMPs, rather it demonstrates the global MMP activity level in a tissue.

Therefore, we performed an *in situ* gelatinolytic assay on tissue sections to measure and localize the activity of MMPs. Although this assay does not differentiate between the two gelatinases, it does provide information of the regional activation that the gelatin zymography cannot. Though we did observe a fluorescent signal in sham-operated animals, this was largely restricted to neuronal cells and blood vessels (Fig. 2.8). This pattern of expression closely resembled the fluorescent immunohistochemistry colocalizing MMP-2 to neurons. In ischemic animals, an increase in the intensity of the fluorescent signal was noted; this can be seen in Figs. 2.8 D-I. No change was observed in the pattern of MMP activity. The *in situ* zymography consistently demonstrated neuronal activity, regardless of time point or treatment.

Figure 2.8. In Situ Zymography. A-C depict sections from a sham-operated, saline-treated animal. This is representative of baseline MMP activity for comparison among experimental groups. Layers 1 and 2 of the cortex do not demonstrate any MMP activity. Restriction to cell bodies is consistent throughout all time points and treatment modalities. A. Magnification 10X; B. 20X; C. 20X in right striatum. D-G. Depict sections through the ipsilateral ischemic striatum (D,E) and the contralateral non-ischemic striatum (F,G) of a tPA-treated ischemic animal 12 hours post-ischemia. Note the difference in the pattern of staining between the two hemispheres. In the ipsilateral striatum, cells appear brighter and areas surrounding cells are more fluorescent in comparison to the contralateral striatum and to the non-ischemic sections seen in panels A-C. Panels H and I depict ipsilateral (H) and contralateral (I) striatal sections in a non-treated ischemic animal. Cells appear intact and the extracellular space does not demonstrate any MMP activation as seen in panels D and E. J. Negative control for these sections. The slide was treated to all of the same conditions as test slides with the exception of incubation with the DQ-labeled gelatin.



Discussion:

In the present study, we found that tPA administration following a thromboembolic cerebral infarct induces the activation of MMP-2 and -9 and that this is associated with increased BBB breakdown in a time-dependent fashion. We demonstrate a significant reduction in infarct volume conferred by thrombolytic treatment with tPA. This result which is widely published (Vivien and Buisson, 2000; Gautier et al., 2003a; Graham, 2003; Yepes et al., 2003) was used here to confirm the reliability of the ischemic model used for this study. Use of TTC staining as a measure of infarct volume has been shown to be equivalent if not superior to cresyl violet staining and hematoxylin-eosin staining; two alternative histological techniques (Bederson et al., 1986; Tureyen et al., 2004). Our goal in the use of TTC as a measure of infarct was not to reproduce previously published reports but rather to confirm the validity of our model. And indeed we observed uniform and reproducible cerebral infarcts. Gelatin zymography experiments show a clear increase in MMP activity following tPA administration. Although it has been previously demonstrated using a similar animal model that MMP-9 was upregulated at 6 h (Aoki et al., 2002) following ischemia, our results are the first to outline the full temporal profile of the combination of tPA administration in a thromboembolic model in the first 24 h of ischemic injury. During the preparation of this manuscript, two reports were published which also outline the activation of MMP-9 following tPA administration. These manuscripts involved the use of an intraluminal suture model of ischemia followed by mechanical reperfusion, and one (Tsuiji et al., 2005), also examined MMP-9 with tPA-knockout mice. The main conclusions of this study

were that tPA administered exogenously increased MMP-9 activation twofold, and the genetic knockout of tPA reduced MMP-9 levels compared to wild-type mice (Tsuji et al., 2005). The second study (Kahles et al., 2005) examined BBB breakdown in the same ischemia-reperfusion (intraluminal suture) model, with the exception that tPA was administered intra-arterially to the MCA. This study found that the effects of tPA on the BBB were enhanced when tPA was incubated with clot material (Kahles et al., 2005). BBB breakdown and MMP activation are dynamic processes following cerebral ischemia and tPA administration, our study fully outlines the 24-h period after ischemic injury, in a model very similar to the clinical situation, using a clot formed from the animal's own blood and administering tPA 1 h after the insult, within the window of opportunity used in human stroke patients. The results of our gelatin zymography experiments show a clear increase in both the pro- and active forms of MMP-9 after tPA administration. Six hours following ischemia, pro-MMP-9 was significantly higher than at any other time point studied, while the active form of MMP-9 was elevated at 6, 12, and 24 h post ischemia. This increase in MMP-9 activity coincides with the greatest amount of loss of laminin and fibronectin in the same tPA-treated animals. tPA treatment of ischemic animals results in significantly greater MMP-2 activity than that observed in ischemic animals receiving saline treatment, although this activation showed no significant change over time. MMP-2 is constitutively bound to the cell membrane by a tissue inhibitor of metalloproteinases (TIMP) and a membrane-type metalloproteinase (MT-MMP). Due to the vulnerable nature of this gelatinase, it was observed that MMP-2 was present at all time points, in all treatment groups, including non-

ischemic, non-treated animals. MMP-2 is not likely to be a major contributor to BBB breakdown in this setting, as sham-operated animals also demonstrated a baseline level of activity from this enzyme. Despite this, an increase in MMP-2 activity following tPA administration could aid in the activation of MMP-9. In terms of its cellular localization, MMP-2 was present most obviously in neuronal cells. Nontreated ischemic animals demonstrated astrocytic expression of MMP-2. However, tPA-treated animals rarely demonstrate astrocytic MMP-2 expression and such expression was restricted to the core of the infarct. The *in situ* zymography data were correlated to that obtained from immunohistochemistry of each of the MMPs. Neuronal cells predominantly demonstrated MMP activation as measured by this non-specific activity assay. Based on our double-immunostaining results, the activated gelatinase is likely to be MMP-2. It is also possible that the activity observed in these sections is due to other MMPs, as MMP-2 and -9 are not the only proteases able to digest gelatin, MMP-3 (stromelysin) and MMP-7 (matrilysin) are also capable (Gasche et al., 2001) and further investigations are underway to elucidate the relative contributions of these and other metalloproteinases. The results of immunohistochemical experiments also outlined the temporal profile of BBB breakdown by examining the loss of structural components laminin and fibronectin. Here, we observed a loss of BBB integrity which followed MMP-9 activation in tPA-treated ischemic animals. Although the loss of laminin and fibronectin was also observed in saline treated ischemic animals, it was not observed until 12 h post-ischemia whereas tPA-treated ischemic animals demonstrated a loss of positive laminin staining which began as early as 6 h post-ischemia. This loss of laminin

coincided with the increase of MMP-9 activity seen in gelatin zymography experiments therefore, it can be said that tPA is associated with the loss of structural integrity by way of increased MMP-9 activation. Sham animals receiving tPA intravenously did not demonstrate an increase in MMP-9 either in the pro-form of the active form, nor did they demonstrate destruction of the BBB. Furthermore, MMP-9 activation was not observed in the contralateral hemisphere of tPA-treated ischemic animals at any time point. This result can be explained by the hypothesis that tPA is only detrimental to the injured brain (Vivien and Buisson, 2000). If the exogenous application of tPA was harmful to the healthy brain, we would expect to see some activation of MMP-9, and breakdown of the BBB in tPA-treated sham-operated animals or in the contralateral, nonischemic hemisphere of tPA-treated ischemic animals, but this was not observed here.

In conclusion, the current study demonstrates that MMP-9 activation is temporally similar to BBB breakdown as demonstrated by a loss of structural components laminin and fibronectin. The MMP-9 activation and time course of degradation of the BBB are restricted to those animals administered tPA following a focal embolic ischemic event. These results were the first to demonstrate the temporal profile of activation of MMP-2 and -9 following tPA administration in thrombotic cerebral ischemia. The results presented here led to the following study involving the inhibition of MMPs using doxycycline in combination with tPA thrombolysis. From this first study, it can be said that early MMP inhibition could be ideal for rescuing the BBB from breakdown caused by increased MMP activation induced by tPA administration. While the initial 48 hours following cerebral

ischemia have been widely studied and there is a growing focus on the beneficial effects of MMP inhibition, I chose to follow up this first study with an examination of the long-term effects of tPA on MMP activation and BBB breakdown. This study also included an examination of doxycycline, a broad-spectrum MMP inhibitor, alone and in combination with tPA thrombolysis.

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Chapter 3:
Thrombolysis with Tissue Plasminogen Activator and
Matrix Metalloproteinase Inhibition with Doxycycline is
Deleterious to the Ischemic Brain

Introduction:

Thrombolytic therapy with tissue plasminogen activator (tPA) has proven effective for acute focal cerebral ischemia. However, the risk of cerebral hemorrhage associated with the use of tPA limits the number of patients eligible for treatment (1995; Sumii and Lo, 2002; Gautier et al., 2003b; Liu KJ, 2005). Activation of matrix metalloproteinases (MMPs) has been previously implicated in the hemorrhagic transformation observed following tPA administration (Hosomi et al., 2001; Cai et al., 2005; Griffin et al., 2005; Rosell et al., 2006). Previously, we and others have shown increased MMP activity following tPA treatment, suggesting a link between the plasmin cascade and MMPs (Sumii and Lo, 2002; Maier et al., 2004; Newby, 2005; Kelly et al., 2006; Zhao et al., 2006b). Specifically, MMP-2 and MMP-9 (gelatinase A and B, respectively) are thought to be largely responsible for blood-brain barrier (BBB) breakdown due to their specificity for extracellular matrix proteins (Maier et al., 2004; Kelly et al., 2006). This BBB breakdown commences the evolution toward hemorrhagic transformation.

Recent research has focused on the inhibition of the gelatinases in an attempt to attenuate breakdown of the BBB following focal cerebral ischemia (Sumii and Lo, 2002; Zhao et al., 2006b). Several synthetic MMP inhibitors have been manufactured but are not suitable for clinical use due to complications with route of administration, toxicity and unknown side effects in humans. Doxycycline (DOXY), a synthetic tetracycline derivative, is used clinically to treat a variety of conditions including rheumatoid arthritis and periodontal disease (Koistinaho et al., 2005). Aside from its antimicrobial action, DOXY has also been shown to possess MMP

inhibitory properties at doses below those used for its antimicrobial action (Golub et al., 1991; Paemen et al., 1996). Due to its clinical approval for other human conditions and its safety, efficacy, and known MMP inhibitory actions, DOXY is a logical compound to test for use in combination with tPA to achieve thrombolysis while retaining integrity of the BBB.

Different models of ischemia report the activation of MMP-9 in the early hours following ischemia, and this activation tends to be exacerbated with the administration of tPA (Mun-Bryce and Rosenberg, 1998; Rosenberg, 1998; Heo et al., 1999; Zhang et al., 2001; Sumii and Lo, 2002; Kelly et al., 2006). MMP-2 on the other hand, has a longer latency to activation and does not reach maximal activation until 5-7 days following ischemia (Cuzner and Opdenakker, 1999b; Gasche et al., 2001). The results displayed in Chapter 2 outline the acute temporal activation of MMP-9 throughout the first 24 hours following focal ischemia with tPA thrombolysis (Kelly et al., 2006). Other studies have limited the end point of observation to within 48 hours of the onset of stroke (Kahles et al., 2005; Rosell et al., 2006; Zhang et al., 2006). For this particular study, a seven-day survival time was chosen to examine the longer term effects of tPA on MMP activation and the combined effect of tPA with DOXY.

It is well-known that plasmin, formed from the cleavage of plasminogen by tPA, is a potent activator of MMPs (Griffin et al., 2005; Rosell et al., 2006). Thus, by inhibiting MMP activation with DOXY and lysing an occlusive blood clot with tPA, the BBB is expected to retain its integrity. Interestingly, we found the combination of tPA and DOXY to be harmful. Combined treatment increased

MMP-2 activity, increased neuronal loss and increased BBB breakdown. To further elucidate the nature of the harmful interaction of tPA with DOXY we used cultured endothelial cells incubated with comparable doses of tPA, DOXY and the combination thereof.

Materials and Methods:

Animal Model

Male Sprague-Dawley rats (locally obtained, 250-300g) were housed in a humidity and temperature-controlled environment with a 12-hour light/dark cycle. Care and use of animals complied with guidelines as outlined by the Canadian Council for Animal Care. Rectal temperature was maintained at 37°C with a thermostat-controlled heating pad (Harvard Apparatus; Holliston Massachusetts, USA), during surgical procedures. Animals were randomly assigned to one of eight experimental groups: Ischemia-Saline-Saline; Ischemia-tPA-Saline; Ischemia-Saline-DOXY; Ischemia-tPA-DOXY; Sham-Saline-Saline; Sham-tPA-Saline; Sham-Saline-DOXY; Sham-tPA-DOXY. Each group included six animals. Animals were anesthetized using inhalant halothane, (3% to induce and 1.5% for maintenance) in 70% oxygen and 30% nitrogen. For this study we used a clinically relevant thromboembolic model of focal cerebral ischemia (Wang et al., 2001; Kelly et al., 2006). Briefly, a surgical incision was made in the ventral neck to expose the common, internal and external carotid arteries. The external carotid artery (ECA) was fully ligated for insertion of a PE-10 catheter (Fisher Scientific, New Jersey, USA). The catheter was advanced through the internal carotid artery (ICA) to the distal opening of the middle cerebral artery (MCA). Blood was drawn from this region and mixed with thrombin

contained in the catheter to form a clot. Clot formation was allowed to progress for 15 minutes and the clot was deposited followed by a small saline injection advancing the clot to the MCA. The catheter was withdrawn after 15 minutes, animals were then returned to their cages for recovery. Those animals randomly assigned to a sham group received the same surgical procedure with the exception of clot formation and deposition. Animals were monitored continuously throughout the recovery period. One hour following sham or ischemic operations, animals received tail vein injection of saline or tPA (10 mg/kg; Genentech, San Francisco, California, USA) in combination with saline or DOXY (45 mg/kg; Sigma-Aldrich, St. Louis, Missouri, USA) subcutaneously (Kaito et al., 2003; Tessone et al., 2005). Daily injections of saline or DOXY (22.5 mg/kg) (Wang et al., 2002; Wang et al., 2003a) were administered subcutaneously for seven days following surgery. On the seventh day, animals were euthanised, brains quickly removed and flash frozen in ice-cold isopentane (Fisher Scientific, New Jersey, USA). Brains were kept at -80°C until use for analysis of infarct volume, BBB breakdown and activation of MMP-2 and -9.

Tissue Preparation

From experimental animals serial coronal sections ($20\mu\text{m}$) through the known area of infarct were used for immunohistochemical analyses. The remainder of the infarcted area was dissected into left and right hemispheres for zymographic analysis.

Immunohistochemistry

Immunohistochemistry was performed with antibodies recognizing neuronal nuclei (NeuN) for quantification of infarct volume and laminin and IgG for

quantification and regionalization of BBB breakdown. Double immunofluorescence was used to co-localize antibodies recognizing MMP-2 and -9 to neurons or astrocytes (anti-glial fibrillary acidic protein [GFAP]). This double fluorescent immunostaining technique was used to determine the specific cell-type expressing the increased MMP activation observed by gelatin zymography. Sections were fixed in formalin, except when antibodies recognizing MMP-2 and MMP-9 were used, and dehydrated through graded alcohols and cleared in xylene. Sections were blocked for endogenous peroxidase and incubated overnight with the primary antibodies: rabbit anti-laminin (Sigma, 1:100); mouse anti-NeuN (Chemicon International, 1:100); rabbit anti-MMP2 (Chemicon International, 1:50); mouse anti-MMP9 (Chemicon International, 1:100); and mouse or rabbit anti-GFAP (DAKO, 1:1000) followed by incubation with appropriate secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA; 1:200). Those secondary antibodies not conjugated to fluorescent chromogens were incubated for 30 mins with an avidin-biotin complex (DAKO, Denmark) and visualized with diaminobenzidine (DAB) (Biomed Corp., USA). Negative controls, sections incubated without primary antibodies, were run with each assay.

Quantification of Neuronal Loss and Infarct Volume Measurements

To assess the ischemic area, sections with positive NeuN immunoreactivity were counted by observers blind to treatment conditions. Four pre-determined regions of interest (ROI) in each ischemic hemisphere were used for quantification, these are shown in Figure 3.1 A. Briefly, the four regions include; dorso-medial striatum, lateral striatum, the dorsal cortex, and lateral cortex, in that order. These four ROIs

were used to count positive neuronal staining throughout the infarct area as illustrated in Figure 3.1A. Unbiased stereological procedures were used. The four areas encompass the entire infarcted region, core and penumbra, and several sections from each brain were analyzed and the average number of surviving neurons was taken from all four regions. For cellular, laminin and IgG extravasation counting was performed at 10X magnification. A conventional method of measuring infarct volume by 2,3,5-triphenyltetrazolium chloride (TTC) staining was not utilized here as animals were retained for seven days following the induction of ischemia, and after 72 hours of ischemia, TTC staining becomes unreliable (Benedek et al., 2006). NeuN immunohistochemistry allowed us to count surviving neurons within the infarcted area. Counting surviving neurons provides a more robust index of ischemic severity as it is a true measure of neuroprotection.

To further confirm the validity of this process, we performed infarct volume measurements on the same 20 μ m coronal sections incubated with the NeuN antibody. The area of ischemic damage was clearly outlined and traced using the lasso tool on Photoshop. Infarct volume was integrated across tissue sections and over the ipsilateral hemisphere. Edema was taken into account by calculating the percent increase in hemisphere size. Data are represented as mean $\pm$ SEM.

Blood-Brain Barrier Breakdown

Laminin Immunohistochemistry

Quantification of laminin immunostaining was performed by counting intact blood vessels (Vosko et al., 2003). Laminin immunostaining in conjunction with IgG extravasation was used as a measure of BBB breakdown, as MMP-2 and -9

specifically target this structural protein. Moreover, evidence suggests that using tracers to evaluate BBB damage could underestimate the extent of BBB permeability, particularly in non-perfused areas (Todd and Butterworth, 1999; Vosko et al., 2003) thus by quantifying the amount of laminin remaining, we were able to examine structural elements of the BBB.

IgG Extravasation

IgG extravasation provides a gross measure of BBB breakdown (Calingasan et al., 1995; Todd and Butterworth, 1999; Kelly et al., 2006) and was used to corroborate the laminin immunohistochemical analysis of BBB breakdown. By incubating slides with species-specific IgG, microscopic visualization and quantification of the extent of leaky blood vessels is possible. Increased permeability of blood vessels was quantified using brightfield microscopy and an unbiased stereological procedure by investigators blind to treatment group. Values obtained in each of the same 4 ROI fields used above for neuronal cell loss and laminin loss analyses, were averaged and means used to determine the extent of BBB damage. Intact blood vessels positive for IgG were counted. Permeable (damaged) blood vessels were apparent with IgG staining in the surrounding parenchyma.

Rat Brain Endothelial Cell Culture

Endothelial cells were isolated from adult rat brain (200-250 g, Sprague-Dawley rats) striatum and cortex. Isolation was done through digestion in 0.1% collagenase at 37°C, and separation on a 15-20% percoll gradient. Cells were collected and cultured in DMEM/10% fetal bovine serum (FBS) and maintained at 37°C in 5%

CO₂/95% ambient mixed air. Culture media was changed every 72 hours for 8-10 days until cells reached 80% confluence.

Endothelial Cell Characterization

Cultured endothelial cells were characterized by immunocytochemistry, conditioned media was removed, PBS was added to wells to rinse cells of any remaining debris. Cells were blocked for any endogenous peroxidase activity by incubation in 0.03% hydrogen peroxide solution, then incubated for at least one hour with primary antibodies used to identify the endothelial cell type. Antibodies used included mouse anti-endothelial nitric oxide synthase (eNOS; 1:500); and mouse anti-rat endothelial cell antigen (RECA; 1:250). Following primary antibody incubation, appropriate secondary antibodies were used followed by incubation with avidin-biotin complex to enhance chromogen signal and finally with DAB as the chromogen.

Experimental protocol for endothelial cells

To examine the effects of tPA on MMP activation in cultured endothelial cells we incubated cells with tPA (20 µg/mL) for 24 hours. This dose of tPA was chosen based on a dose-response curve in the literature. The authors used a range of doses of tPA from 5 µg/mL to 40 µg/mL, they determined that 10-20 µg/mL was not toxic to cultured cells but did stimulate cells to activate downstream proteolytic cascades (Liot et al., 2006). Cells in medium without tPA were used as naïve controls and cells incubated with PBS as vehicle were used as controls. After the 24 hour incubation period, the activity levels of MMP-2 and -9 were determined by gelatin zymography.

Gelatin Zymography

Brain tissue was homogenized in lysis buffer (50mM TrisHCl; 150mM NaCl; 5mM CaCl_2 ; 0.05% Brij-35 and 1% v/v triton-X 100). Total protein concentration was determined according to Bradford's protocol. Samples were equilibrated with gelatin-Sepharose 4B (Pharmacia Biotech, USA), washed with binding buffer (50mM TrisHCl; 150mM NaCl; 5mM CaCl_2 ; 0.05% Brij-35) and elution buffer (50mM TrisHCl; 150mM NaCl; 5mM CaCl_2 ; 0.05% Brij-35 and 10% DMSO). Equal amounts of sample protein were separated on 8% acrylamide gel containing 0.1% gelatin. Purified human MMP-2 and -9 standards (Oncogene; $1\mu\text{g}/\mu\text{L}$) were loaded together in one lane. Following electrophoresis, gels were incubated in renaturing buffer (2.5% v/v triton-X 100) for one hour. Gels were then equilibrated in incubation buffer (50mM TrisHCl; 0.15M NaCl; 5mM CaCl_2) for ten minutes followed by incubation in fresh incubation buffer at 37°C until bands became apparent with Coomassie blue R-250 stain. Gelatin zymography was performed three separate times for each hemisphere.

Gelatin Zymography of Conditioned Media

Gelatin zymographic experiments were performed as above, with the use of conditioned media instead of homogenized brain tissue. However, to identify the true gelatinolytic nature of the bands observed, gels were incubated with incubation buffer supplemented with 1mM EDTA, 100 mM DOXY, or $10\mu\text{M}$ N,N,N',N' -tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (Lalu et al., 2003; Lalu et al., 2006).

Gelatinolytic activity appeared as clear bands on a blue background. Images of gels were captured by scanning each gel on an HP flatbed scanner; luminosity of bands were quantified with ImagePro Analysis System. Results of all zymographic experiments are provided as an average luminosity of the bands of the three separate trials.

Statistical Analysis

Quantitative data are expressed throughout as mean $\pm$ standard error of the mean (SEM). Statistical comparisons for infarct size, laminin immunostaining, IgG extravasation and zymographic luminosity were performed using one-way ANOVA followed by Tukey post-hoc analysis. Differences with $p < 0.05$ were considered statistically significant.

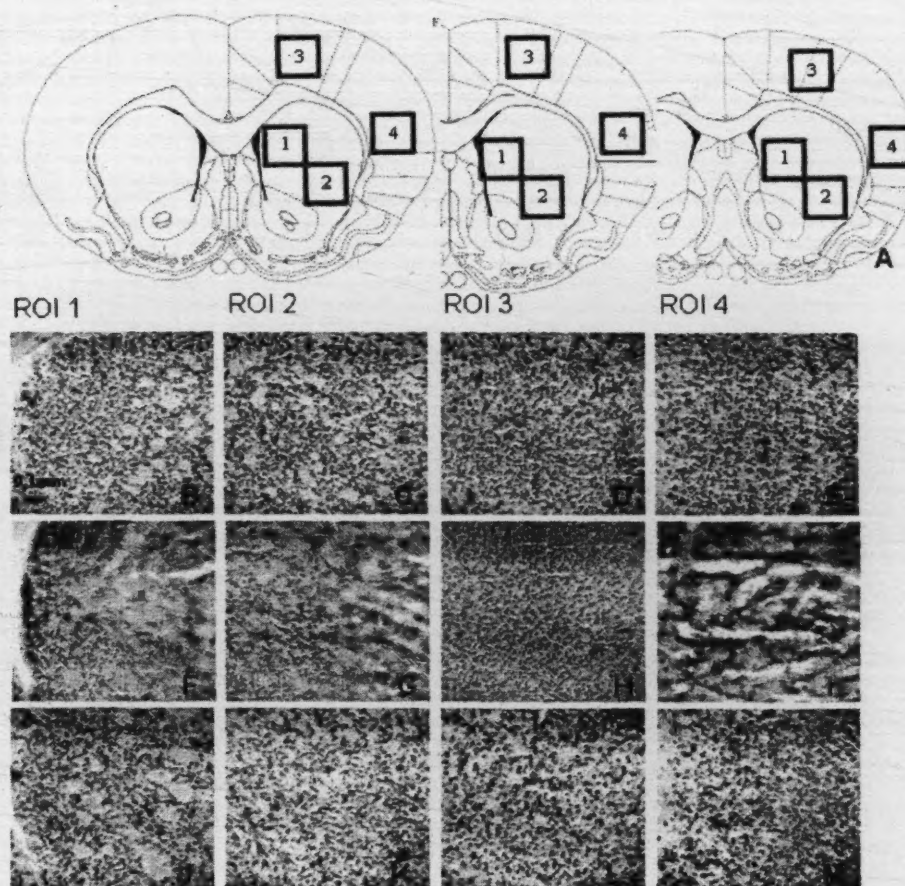
Results:

Quantification of Neuronal Loss and Infarct Volume

Seven days following ischemic injury, animals that underwent both thrombolysis with tPA alone, and in combination with DOXY demonstrated increased neuronal loss compared to vehicle treated rats (Respectively, 227.9 ± 30.76 ; 193.5 ± 26.37 ; 175.8 ± 26.89 ; values represent number of NeuN positive immunostained neurons; Figure 3.1 B-M). Sections obtained from sham-operated animals are displayed in Figure 3.1 B-E, and show a large population of neuronal cells throughout all 4 ROIs. Figure 3.1 F-I display representative sections obtained from animals treated with tPA alone. This series of photomicrographs shows clearly the increased neuronal loss in tPA-treated animals compared to sham-operated animals. Photomicrographs J-M in Figure 3.1 display representative sections from an ischemic animal treated with

DOXY alone. The dramatic loss of neurons observed in animals receiving combination therapy is not present in these same regions of interest. Treatment with DOXY alone resulted in the protection from neuronal loss compared to vehicle-treated ischemic animals and ischemic animals treated with a combination of tPA and DOXY (Respectively, 294.3 ± 23.7 ; 175.8 ± 26.89 ; 193.5 ± 26.37 ; Figure 3.1N).

Figure 3.1. Neuronal Survival Measurement. A. Coronal sections represent regions of interest (ROI) used to count positive neuronal staining throughout the infarcted area (Paxinos and Watson, 1998). B-E. Photomicrographs represent a sham animal treated with tPA and saline. F-I. Series represents ischemic animals treated with tPA and saline. Note in F, G and I loss of neuronal staining, in comparison to the other two groups displayed. J-M. Represent a DOXY-treated ischemic animal. Neuronal survival is similar to sham-operated animals. N. (see page 116) Histogram depicts the percentage of neuronal loss in all study groups.



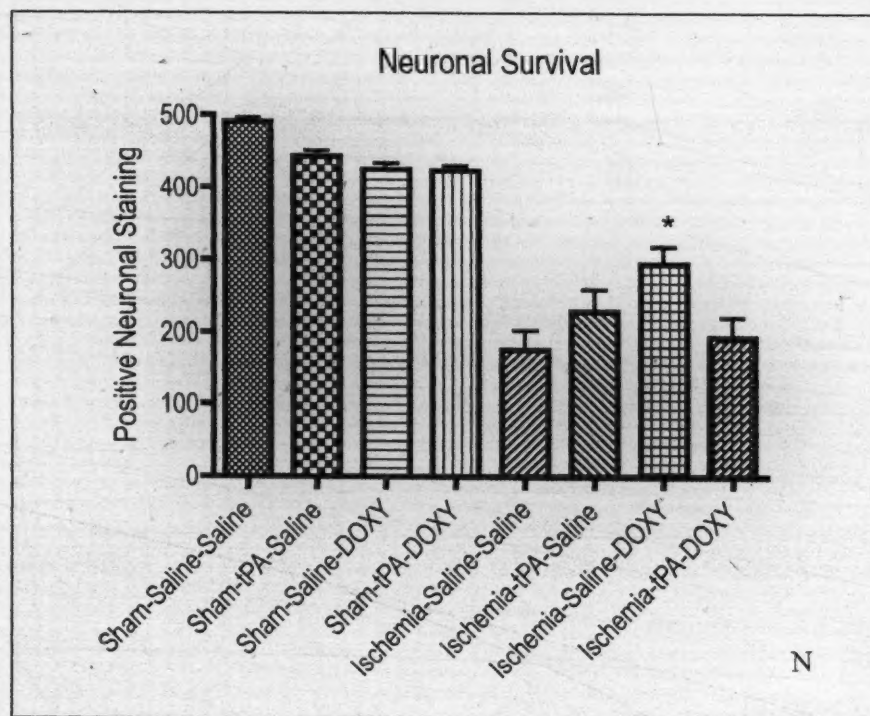
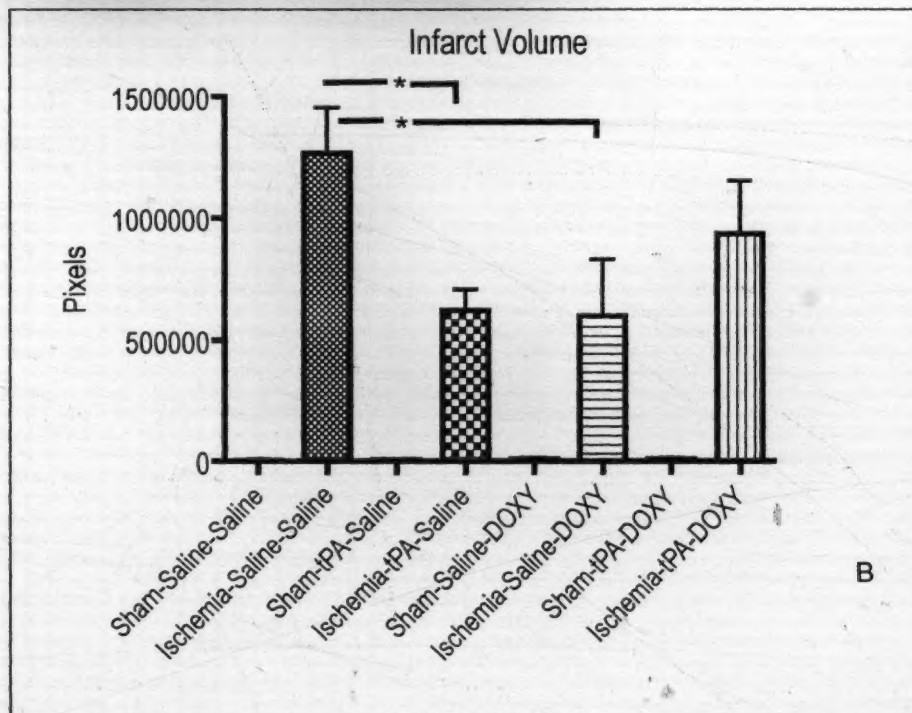
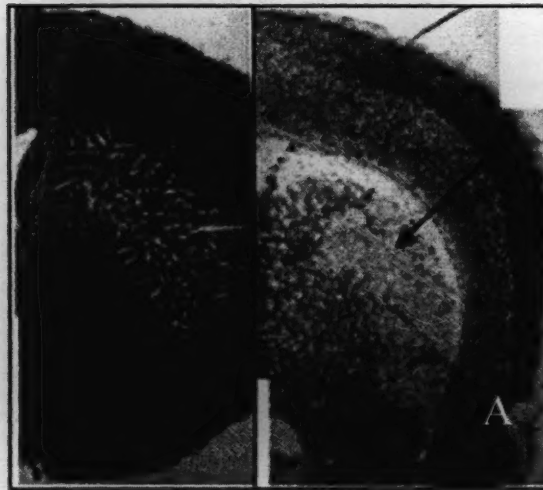


Figure 3.2 A displays an example of the infarcted area that was traced and quantified using pixel density within the defined area. The data obtained from this volume measurement corroborated that obtained by neuronal cell counts. ANOVA showed a significant reduction in the infarct volume in tPA-treated animals and DOXY-treated animals (623205 ± 84402 ; 601065 ± 229379 ; numbers represent pixel density $\pm$ SEM). Overall results are displayed in Figure 3.2 B.

Figure 3.2. Infarct volume measurement. A. Low magnification photomicrograph of a coronal section with a NeuN antibody. The ischemic area is clearly identifiable and can be measured using standard infarct volume measurement techniques. Multiple sections were analysed and included in calculations for each treatment group. B. Histogram depicts all infarct volume data.

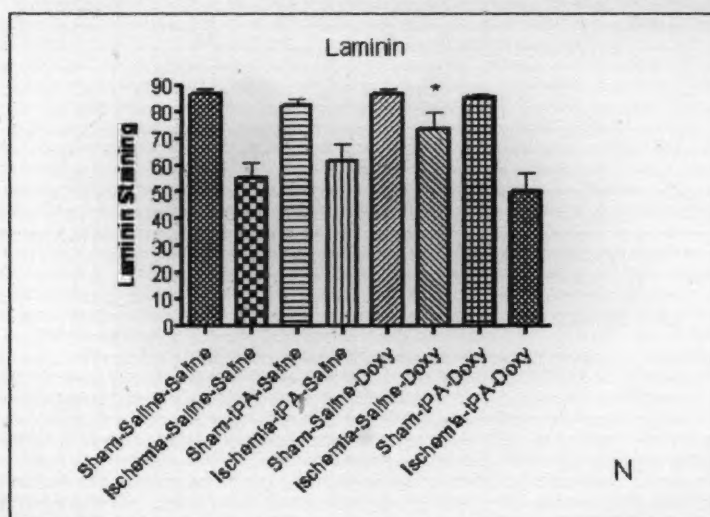
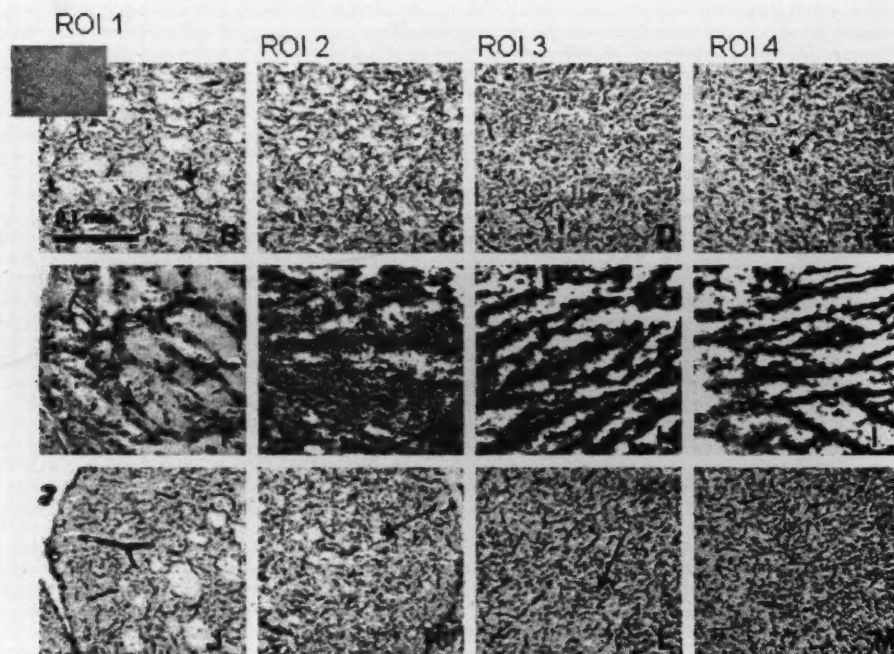
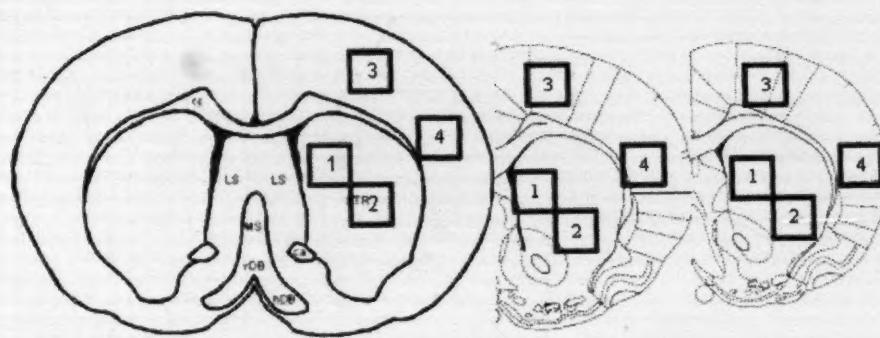


Blood-Brain Barrier Breakdown

Laminin Immunohistochemistry

Laminin immunoreactivity in sections obtained from ischemic animals treated with DOXY alone was similar to sham-operated animals (73.3 ± 6.3 . Values represent the number of intact blood vessels positive for laminin $\pm$ SEM). There was more laminin immunoreactivity in the DOXY alone treatment group than in either the vehicle-treated group or the group receiving combination therapy (Figure 3.3N). Vehicle-treated ischemic animals and animals treated with the combination of tPA and DOXY showed the greatest degree of laminin loss (54.98 ± 5.97 and 50.32 ± 6.7 , respectively). Interestingly, those ischemic animals receiving combination therapy demonstrated a greater loss of laminin than vehicle-treated ischemic animals (Figure 3.3). This increased loss of laminin translates to a greater degree of BBB breakdown.

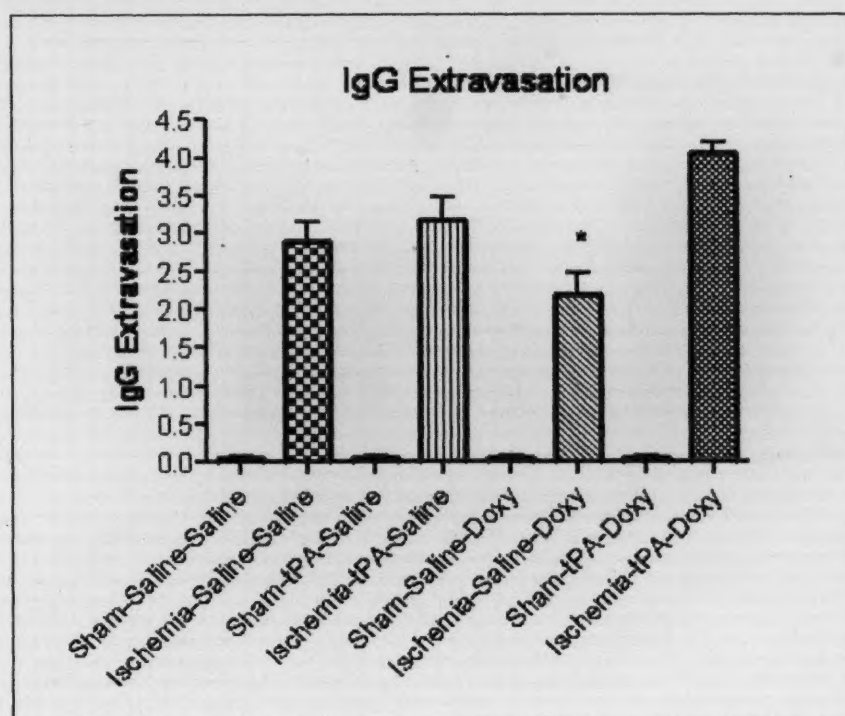
Figure 3.3. Laminin Immunohistochemistry. A. Coronal section represent the 4 ROIs used to count positive laminin staining as a measure of BBB breakdown (Paxinos and Watson, 1998). Each of the four regions was counted in three consecutive sections as in Figure 1. B-E. Represent sham-operated, untreated animal. Note intact appearance of blood vessels. Inset represents negative control. F-I. Represent an ischemic animal treated with combination of tPA and DOXY. Note the absence of laminin staining and the broken appearance of blood vessels in F as highlighted by arrow. J-M. Represent an ischemic animal treated with saline and DOXY. Note the difference in BBB destruction between the three groups depicted. N. Histogram depicts the amount of laminin loss in ischemic and sham animals. DOXY treatment alone is significantly more protective of the BBB than any other treatment tested.



IgG Extravasation

Ischemic animals treated with DOXY alone exhibited the least BBB destruction (2.18 ± 0.28 ; values represent extent of IgG extravasation) as measured by the leakage of IgG from cerebral blood vessels (Figure 3.4). This was significant compared to all groups tested. Animals treated with the combination of tPA and DOXY demonstrated the greatest degree of IgG extravasation and thus BBB breakdown (4.06 ± 0.14), even more so than untreated ischemic animals and tPA-treated animals (2.9 ± 0.2 ; 3.1 ± 0.30 , respectively). The results of IgG extravasation experiments corroborate the laminin immunohistochemical analyses and confirm a reduction of damage to the BBB conferred by DOXY treatment alone.

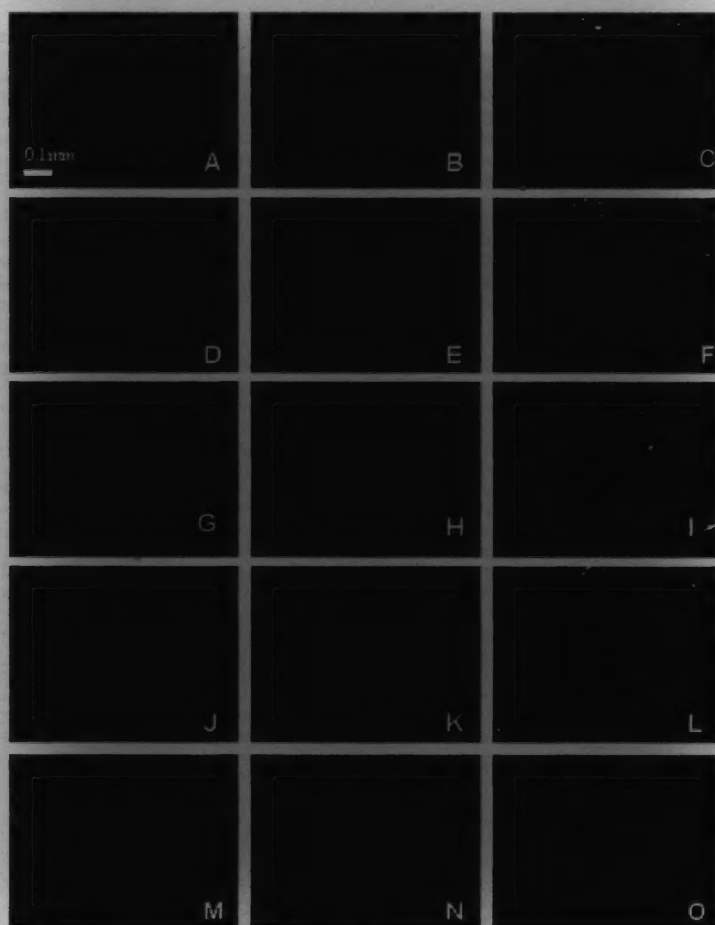
Figure 3.4. IgG Extravasation. Histogram representing IgG extravasation as a measure of BBB breakdown. Scale from 1-5 created as a measure of the extent of IgG extravasation. Destruction of the BBB was also quantified in three consecutive sections through the entire ipsilateral hemisphere as outlined in Figures 3.1 and 3.3. Extent of IgG extravasation to the surrounding parenchyma was given a score on a scale of 1 to 5. The gross measurement of BBB breakdown served to corroborate laminin immunohistochemical data.



Cellular Localization of MMP-2 and -9

Double fluorescent immunohistochemistry identified the specific cellular expression of MMP-2 and -9. Our results demonstrate the ubiquitous neuronal expression of MMP-2 in sham-operated animals (Figure 3.5 A). Interestingly, in all ischemic animals MMP-2 expression was co-localized to astrocytes in the ipsilateral hemisphere. This was apparent in the area surrounding infarcted tissue, where there were few or no viable neurons (Figure 3.5 B). In the contralateral hemisphere, MMP-2 expression was observed to be similar to sham-operated animals, where MMP-2 was localized exclusively to neuronal cells. There was no observed treatment effect on the cellular expression of MMP-2. In contrast, MMP-9, which is not constitutively expressed in the brain, was not observed in any of the treatment groups (7 days post-ischemia). Figure 3.5 J-O depicts the overlay images of MMP-9 immunostaining with NeuN and GFAP.

Figure 3.5. Double immunofluorescent staining to determine specific cellular expression of MMP-2 and -9. A, D, J. Depict NeuN staining, with neuronal cells in green. B, E, H. Depict immunostaining for MMP-2 in red. C. The merged image for a section taken from a sham-operated untreated animal. MMP-2 is co-localized solely to neuronal cells. F. Merged image. Here, MMP-2 is mostly co-localized to neuronal cells however; it is also present in other cells morphologically similar to astrocytes or neuronal processes. These sections are from an ischemic animal treated with DOXY alone. G. Depicts GFAP immunostaining, a marker for astrocytes, in green. I. Represents the merged image. In this untreated ischemic animal, as was observed in all ischemic animals, surrounding the area of infarction, there is an obvious expression of MMP-2 in astrocytes. Therefore, MMP-2 is normally, constitutively expressed by neuronal cells, but following an ischemic event, astrocytes begin to also express MMP-2 in the area surrounding the infarct. K. Depicts MMP-9 immunostaining in red. L. The merged image confirms there is no neuronal or glial expression of MMP-9. M. Immunostaining for GFAP in green. N. Depicts immunostaining for MMP-9. O. The merged image. There is no astrocytic expression of MMP-9. This expression is consistent throughout all animals included in this study, in both contralateral and ipsilateral hemispheres.



Gelatin Zymography

Seven days following cerebral ischemia, MMP-2 was inhibited in ischemic animals treated with DOXY (93.69 ± 10 ; values represent luminosity of bands $\pm$ SEM), while combined treatment of DOXY with tPA did not demonstrate this same degree of MMP-2 inhibition (215.3 ± 9.92). In fact, the combination treatment increased MMP-2 activation beyond levels observed in vehicle-treated ischemic animals (181.2 ± 15.84). Treatment with tPA alone, increased MMP-2 activation following ischemia (216.1 ± 16.65) compared to MMP-2 activation seen in the saline-treated ischemic animals (181.2 ± 15.84 ; Figure 3.6). Representative zymograms and gel luminosity data are depicted in Figure 3.6 A-C. No changes in either the pro- or active-form of MMP-9 were observed in any treatment group.

Gelatin Zymography of Conditioned Media

Conditioned media obtained from cultured endothelial cells incubated with or without tPA was examined for MMP activity. Results from these experiments showed a constitutive presence of MMP-2 in all media tested and a tPA-dependent MMP-9 expression and increase in MMP-2 expression. To confirm we were truly evaluating MMP and tPA-induced MMP activity, during the gelatinase assay we supplemented the incubation buffer with the known inhibitors of gelatinases EDTA (protease inhibitor), DOXY (MMP inhibitor), or TPEN (zinc chelator) (Figure 3.7). No bands were observed in zymograms incubated with EDTA, and TPEN confirming blocked MMP-2 and -9 gelatinolytic activity. Interestingly, DOXY did not inhibit the gelatinolytic activity of tPA-treated media suggesting the interaction between tPA and DOXY affects MMP activation.

Figure 3.6. Representative gelatin zymography. A. Zymogram, demonstrating an increase in MMP-2 activation in untreated ischemic animals, ischemic animals treated with tPA, and ischemic animals treated with a combination of tPA and DOXY. Of note is the reduction of MMP-2 activity in ischemic animals treated with DOXY alone. The luminosity of these bands appears to have returned to a similar luminosity as seen in all sham-operated animals. Note the absence of a band at the level of 92 kDa which would represent the gelatinolytic activity of MMP-9. The level at which we would expect to see MMP-9 expression and activity can be seen in the standard lane. B. Histogram depicting the MMP-2 band luminosity from all gels studied. Asterisk represents group which reached statistical significance. DOXY treatment alone significantly reduced MMP-2 compared to all other ischemic groups. C. Histogram represents MMP-9 band luminosity from all gels studied. No significant difference between any treatment group studied for both pro- and active-form of MMP-9.

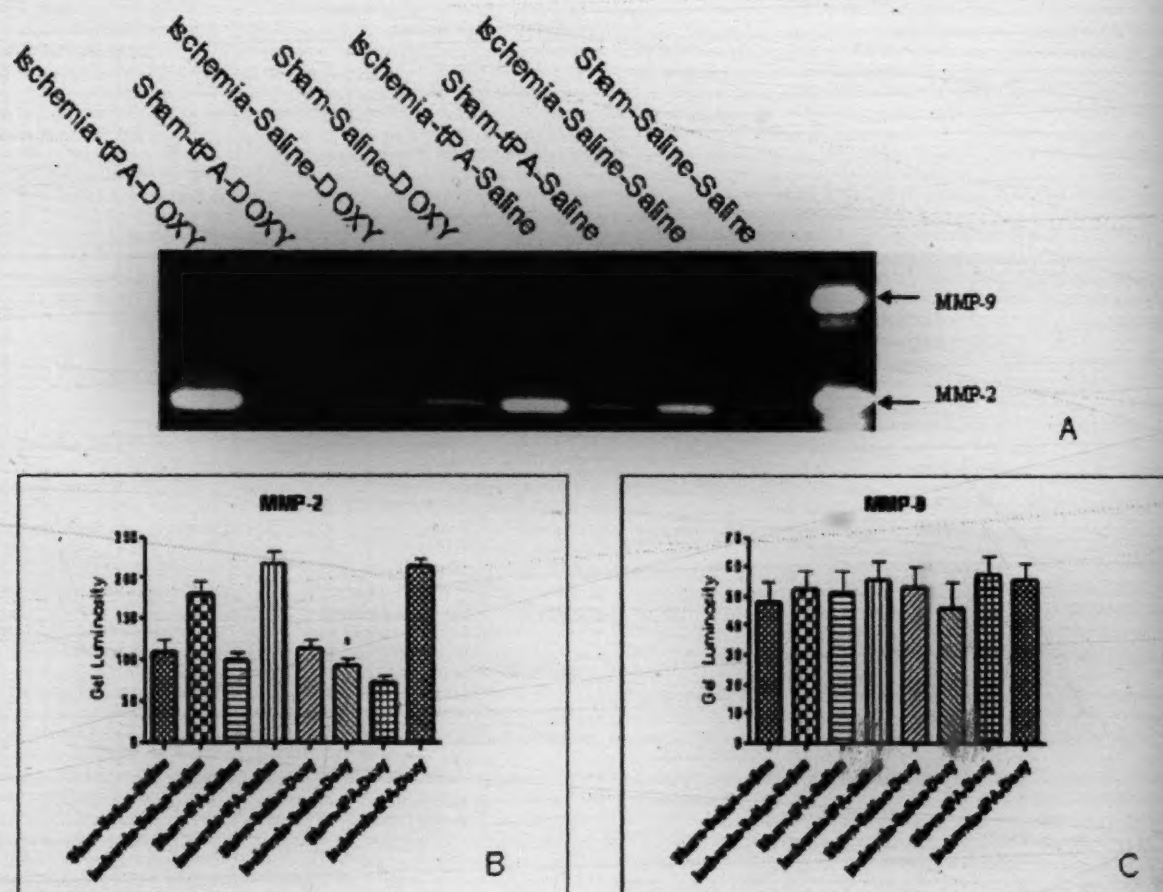
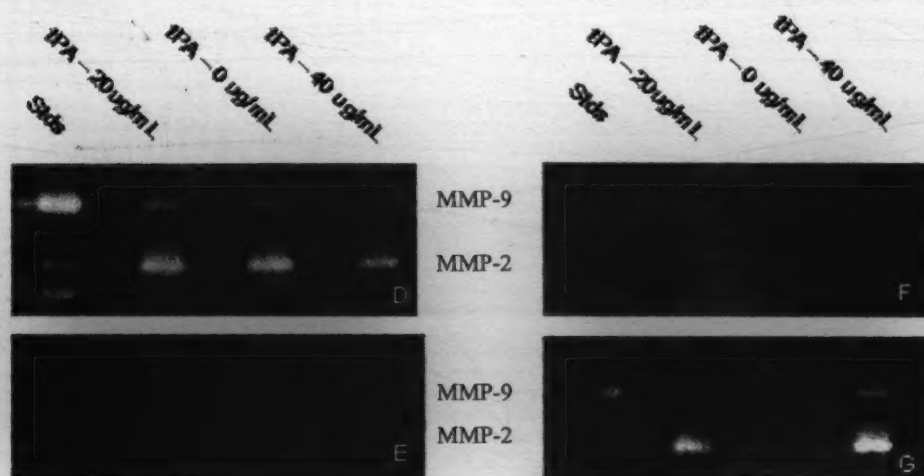


Figure 3.7 Gelatin Zymography of tPA-treated media. A. Representative zymogram of conditioned medium from endothelial cell culture experiments. Cultured endothelial cells were incubated with 0 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$ of tPA for 24 hours before conditioned was collected for gelatin zymography. B. Representative zymogram from experiment of the same conditioned medium though gels were incubated with 1mM EDTA within standard incubation buffer. All gelatinolytic activity is abolished. C. Representative zymogram from the same conditioned medium, gels were incubated with 10 μM TPEN, a specific Zn^{2+} chelator. Note that all MMP activity was again abolished. G. Representative zymograph from experiment using the same conditioned medium though gels were incubated with 100 μM DOXY. The only MMP activity this incubation was able to eliminate were in lanes loaded with conditioned media from culture wells incubated with 0 $\mu\text{g/mL}$ of tPA.



Discussion:

The focus of the previous chapter was primarily on the acute effects of tPA-induced activation of MMP-9 this study examines the same parameters of BBB breakdown and MMP activation seven days following an ischemic event. Thus, MMP-2 is highlighted in this study as it has a longer latency to activation. In the present study DOXY treatment alone reduced laminin loss and IgG extravasation thereby reducing damage to the BBB in the ischemic model. DOXY alone was also the only treatment that reduced overall MMP-2 activity and the amount of neuronal loss throughout the ischemic hemisphere. Combined treatment of tPA and DOXY increased MMP-2 activity and neuronal loss which corroborated increased BBB breakdown as shown by reduced laminin immunostaining and increased extravasation of IgG.

This study is the first to investigate the combination of tPA thrombolysis with MMP inhibition (using DOXY) seven days following a thromboembolic stroke. Our results show that DOXY alone was a superior neuroprotective agent and reduced BBB damage compared to either tPA alone or tPA in combination with DOXY. The combination of tPA with DOXY increased MMP-2 activation and BBB permeability suggesting a potentially detrimental interaction between the two compounds.

While thrombolysis with tPA did reduce neuronal loss compared to vehicle-treated animals, DOXY alone was a superior neuroprotective agent as measured by both neuronal survival and infarct volume. The increased infarct size and neuronal loss observed following combination treatment can be explained by way of the interaction between the plasminogen-plasmin cascade and the proteolytic effect of

MMP (Cuzner and Opdenakker, 1999a; Mun-Bryce et al., 2002; Sumii and Lo, 2002). The mechanism by which MMPs become activated has been known for many years (Cuzner and Opdenakker, 1999a; Mun-Bryce et al., 2002). Most often, MMPs are secreted as inactive zymogens, and are activated in a process known as the "cysteine-switch". Here, the pro-domain, which shields the catalytic site, is cleaved either by proteases, such as plasmin, or by other MMPs (Rajagopalan et al., 1996; Hartung and Kieseier, 2000) causing the pro-domain to breakdown, hydrolyzing the Zn^{2+} ion. Recent evidence in other organ systems, has shown that MMP-2 and -9 can become activated without complete cleavage of this catalytic site, as in situations of oxidative stress (Rajagopalan et al., 1996; Okamoto et al., 1997; Okamoto et al., 2001). Specifically, peroxynitrite oxidizes the sulphydryl bond in the cysteine group, allowing hydration of the catalytic site, rendering the enzyme active while remaining in a pro-enzyme conformation (Okamoto et al., 1997). Plasmin could activate MMPs via this indirect route leaving the Zn^{2+} ion shielded from inhibition by DOXY, which occurs through the chelation of the Zn^{2+} ion. Thus, increased plasmin activation by administration of exogenous tPA would be expected to yield increased MMP activation leading to increased damage of the BBB and combining tPA with DOXY would exacerbate this effect. This is precisely what our data demonstrate and our *in vitro* results further validate this point. Using *in vitro* inhibition of gelatinolytic activity we confirmed the activity of MMP-2 and MMP-9. Incubation buffer supplemented with DOXY did not inhibit the gelatinolytic activity of conditioned media treated with tPA. This result suggests that tPA likely changed the conformation of MMP-2 and -9 in such a way that DOXY was unable to inhibit

the gelatinolytic activity. This suggestion is supported by our data showing that incubation with TPEN, a specific Zn^{2+} chelator inhibited tPA-induced gelatinolytic activity. These data show that DOXY does not chelate the Zn^{2+} ion after tPA-induced MMP activation. In other reports, similar experimental methods were used to confirm that peroxynitrite activates MMPs through an alternative pathway (Lalu et al., 2004). This alternate pathway serves to activate the gelatinases while retaining the pro-form conformation with a shielded active site (Gu et al., 2002). DOXY is thought to act by non-competitive inhibition, in which the inhibitor and the MMP enzyme undergo a conformational change rather than a complete removal of the Zn^{2+} ion (Smith et al., 1999). If plasmin is able to render MMPs active while retaining the pro-enzyme conformation, DOXY would not be able to chelate the Zn^{2+} ion which is part of the active site.

In conclusion, this study is the first to show that DOXY treatment alone is an effective inhibitor of MMP-2 and reduced BBB permeability seven days following focal cerebral ischemia. We have also shown the neuronal expression of MMP-2 as well as glial expression of MMP-2 in regions of infarct following ischemia. Despite short-term clinical evidence which shows tPA to be functionally beneficial, this may not be so in the long term; the activation of MMP-2 after several days serves to amplify the vulnerability of the BBB following cerebral ischemia. Theoretically, the use of tPA as a thrombolytic agent in combination with a broad-spectrum MMP inhibitor should return blood flow to the ischemic area while reducing the amount of BBB damage that occurs following the increased MMP activation induced by tPA. However, we have shown that this combination is not beneficial when administered

in the long term and that after seven consecutive days of dosing, DOXY treatment alone may actually be a superior neuroprotective agent compared to tPA treatment alone due to the effects on the BBB. Thrombolysis with tPA is required in the early hours following a stroke to restore blood flow to the ischemic area. MMP inhibition however, should perhaps be considered as a therapy to be used after blood flow has returned to the area of ischemic damage. The actions of tPA go far beyond fibrinolysis and thus are subject to interact with numerous other pathways triggered during ischemia. From this, we can further explore the mechanistic interactions of tPA and DOXY using an endothelial cell culture model. Using data obtained from this *in vivo* study, and various inhibitors of MMP activation and plasmin activity, we can determine how the tetracycline derivative, DOXY interacts with tPA to enhance the permeability of the BBB.

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Chapter 4:
Molecular mechanisms of tPA and DOXY mobilization of
VEGF

Introduction:

The blood-brain barrier (BBB) is a physical barrier between circulating blood and the CNS (Gaillard et al., 2001; Jodoin et al., 2003; Ma et al., 2005; Schiera et al., 2005; Chan et al., 2006). The barrier is formed by endothelial cells lining cerebral blood vessels and tight junction proteins (TJPs) between endothelial cells (Parkinson and Hacking, 2005; Schiera et al., 2005). These tight junctions consist of the transmembrane proteins claudin and occludin and the family of cytoplasmic proteins, zona occluden (ZO)-1, ZO-2 and ZO-3 (Parkinson and Hacking, 2005). Following an ischemic event such as stroke, the BBB becomes compromised through the action of both intracellular and extracellular proteolytic cascades (Burggraf et al., 2007). This can result in the extravasation of immune cells and proteases to the brain parenchyma, eventually leading to a secondary neurological injury (Parkinson and Hacking, 2005).

Tissue plasminogen activator (tPA), currently the only approved therapy for the treatment of acute ischemic stroke, is associated with an increased risk of hemorrhagic transformation which involves destruction of the BBB. With tPA administration the zymogen, plasminogen, is converted to the active enzyme, plasmin (Wang et al., 2003b). Both plasmin and tPA itself have been shown to activate other serine proteases known as matrix metalloproteinases (MMPs) both *in vitro* and *in vivo* (Wang et al., 2003b; Kelly et al., 2006). These proteases are thought to be partially responsible for BBB breakdown.

Aside from their deleterious proteolytic activity, MMPs have also been related to angiogenic remodeling and migration of neuroblast cells toward areas of

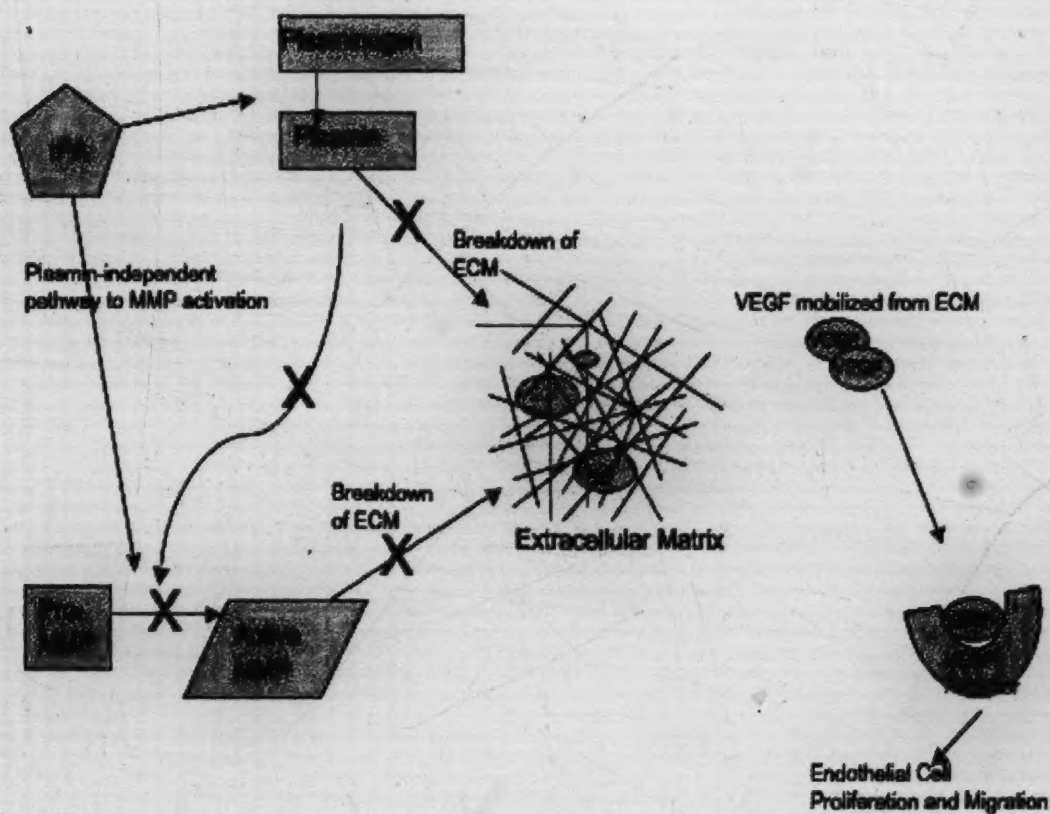
damaged brain (Bergers et al., 2000; Karagiannis and Popel, 2006; Lee et al., 2006b). While inhibition of MMP activation has been shown in several studies to be neuroprotective, it may not always be beneficial, as in the case of angiogenesis and the formation of collateral circulation to peri-ischemic areas. Tetracycline derivatives, minocycline (MINO) and doxycycline (DOXY), have been used extensively for their MMP inhibitory properties, independent of their antimicrobial abilities (Golub et al., 1983; Golub et al., 1987; Golub et al., 1998; Yrjanheikki et al., 1999; Lee et al., 2004; Lee et al., 2006a; Machado et al., 2006; Burggraf et al., 2007). In several studies of carcinogenesis, tetracycline derivatives have been useful, particularly for their ability to inhibit MMP-directed vascular endothelial growth factor (VEGF) stimulation (Pepper, 1997; Lamoreaux et al., 1998; Fife et al., 2000; Zhang et al., 2000; Rudek et al., 2002; Qi et al., 2003; Lee et al., 2004; Lee et al., 2006a). The association between VEGF and the VEGF receptor is critical in the switch to angiogenesis from a quiescent state (Guo et al., 2001). Activation of MMP-9 may increase the availability of VEGF as VEGF is sequestered in the extracellular matrix (Bergers et al., 2000) see Figure 4.1.

The study discussed in Chapter 3 examined the effects of combining tPA and DOXY in an attempt to rescue breakdown of the BBB following thrombolysis with tPA (Kelly et al., 2007; under peer review). This study found that the combination of tPA and DOXY was deleterious to the ischemic brain and increased infarct size while increasing BBB breakdown through the upregulation of MMP-2 activity. Another interesting observation of this study was an increase in the formation of blood vessels on the superolateral surface of the ipsilateral ischemic hemisphere in

animals treated with DOXY, either alone or in combination with tPA (data not shown). This observation, combined with many conflicting studies in the literature examining the effects of DOXY on angiogenesis, led the investigation into the mechanisms underlying the interaction between DOXY and tPA on cerebral endothelial cells. The conversion of plasminogen into the active plasmin has been shown to be accelerated with DOXY in culture, this would be expected to increase the rate of not only MMP activation but also of extracellular matrix breakdown and presumably, the mobilization of VEGF.

While it is recognized that cerebral endothelial cell culture alone is not a model of the BBB, it is a preliminary examination of the signaling mechanisms which would directly affect cerebral endothelial cells, a cell type which is functionally distinct from the endothelium of other organ systems (Lee et al., 2004).

Figure 4.1. Proposed pathway from tPA administration to VEGF mobilization from the extracellular matrix. Each black 'x' represents an area where we were able to manipulate the pathway to halt the activation of the following step. The 'x' on the figure between plasmin and the extracellular matrix, represents the use of α_2 -antiplasmin for the inhibition of plasmin. The 'x' on the figure between the pro-form and the active form of the MMP represents the use of DOXY and MINO as broad-spectrum MMP inhibitors. This inhibition of the conversion to the active form of MMP would inhibit breakdown of the extracellular matrix.



Using primary cultured rat cerebral endothelial cells (RCECs), we show that DOXY suppressed the release of VEGF to the media and that tPA stimulated this release, through the increased activation of MMPs. This effect of tPA was further confirmed and was shown to occur through a plasmin-independent mechanism using an inhibitor of plasmin, $\alpha 2$ -antiplasmin. The increased activation of MMP-9 was also inhibited by minocycline (MINO) another tetracycline derivative with MMP inhibitory properties, although there was no effect on the mobilization of VEGF.

While it is known that VEGF is a stimulatory angiogenic protein, it is not known how tPA alone, or in combination with an MMP inhibitor affects the signaling of VEGF and its release from the extracellular matrix.

Materials and Methods:

Rat Brain Endothelial Cell Culture

All experimental procedures were performed in adherence to the guidelines of the Canadian Council on Animal Care (CCAC) and were approved by the University of Alberta Health Sciences Laboratory Animal Services.

Male Sprague-Dawley rats (200-250g) were euthanized and brains removed. Rat brain endothelial cells were obtained from the grey matter using the method outlined by Abbott et al. (1992). Brains were removed and placed in ice-cold Hanks' Balanced Saline Solution (HBSS). In a laminar flow hood, cerebellum, brain stem and meningeal layers were removed and discarded. Cortex and subcortical grey matter (striatum) were isolated and returned to fresh HBSS. All grey matter was gathered and chopped with a scalpel into pieces less than 1 mm³. Brain bits and HBSS were centrifuged and supernatant removed. The pellet was then resuspended

in 0.1% collagenase containing 1% fetal bovine serum (FBS), and incubated at 37°C for 30 minutes for digestion with shaking. Once digested, the tissue was triturated five times, rinsed of collagenase solution and resuspended in Dulbecco's Modified Eagle's Medium (DMEM). Endothelial cells were isolated by centrifugation on a 20-25% Percoll gradient. Finally, cells were plated onto culture wells pre-coated with calfskin collagen and maintained at 37°C in 5% CO₂. When cells reached 70-80% confluence, the medium was changed to DMEM containing 10% FBS supplemented with one of the following drug cocktails: tPA (20µg/µL), DOXY (10µM), tPA and DOXY combined, MINO (10µM), tPA and MINO combined, α₂-antiplasmin (150 nM) alone, tPA + α₂-antiplasmin, DOXY + α₂-antiplasmin, tPA + DOXY + α₂-antiplasmin. In all conditions, controls were incubated with media supplemented with the vehicle of drug treatment for tPA, DOXY and MINO experiments, this vehicle was phosphate-buffered saline (PBS) and for all experiments with α₂-antiplasmin, this was 0.1% DMSO. Cells were incubated under each condition for 24 hours before conditioned media and cell lysates were collected.

Characterization of Endothelial Cells

Endothelial cells to be utilized for characterization purposes were grown to 70-80% confluency on collagen-coated 24-well plates containing cover slips, as outlined above. Media was removed, and cells were fixed in 4% formalin for five minutes. Endogenous peroxidase activity was inhibited by incubation with 0.03% hydrogen peroxide and 50% methanol. Non-specific antibody binding was blocked by incubation with 10% normal horse serum (NHS) and a solution of PBS with 0.25% triton-X 100. Cells were then incubated overnight at 4°C with one of the following

primary antibodies: anti-eNOS (1:500), anti-RECA (1:1000), or anti-GFAP (1:1000). Wells designated negative controls were subject to all conditions with the exception of incubation with primary antibody. Secondary antibodies were specifically targeted to the host of the primary antibody and were incubated for 30 minutes at room temperature. Cells were then incubated with avidin-biotin complex to enhance the signal of the primary antibody and finally, 3,3'-diaminobenzidine (DAB) was added for colorimetric confirmation. Coverslips were then removed from plates and mounted onto slides using permount.

Gelatin Zymography

Conditioned media was subject to MMP extraction procedure as outlined by Oh et al., 1999. Briefly, media was incubated with gelatin sepharose beads for 1 hour, washed in binding buffer (50mM TrisHCl; 150mM NaCl; 5mM CaCl₂; and 0.05% Brij-35), and washed in elution buffer (50mM TrisHCl; 150mM NaCl; 5mM CaCl₂; 0.05% Brij-35 and 10% DMSO). Equal amounts of protein were then separated on 8% acrylamide gels containing 0.1% gelatin at 150V for 1.5 hours at 4°C. Following electrophoresis, gels were washed for 1 hour in 2.5% (v/v) Triton solution to rid gels of SDS. Gels were then incubated for 24 hours at 37°C in incubation buffer (50mM TrisHCl; 0.15M NaCl; 5mM CaCl₂). Following this incubation, gels were stained with Coomassie blue R-250 for 1 hour and destained in destaining solution containing methanol and acetic acid until clear bands were apparent on a blue background. The gelatinolytic activity of these bands was confirmed by adding 0.5 mM APMA, 10mM EDTA or 100mM DOXY to the incubation buffer.

Western Blotting

For conditioned RCECs, media was removed and retained for gelatin zymography as described above. Culture plates were washed twice with PBS and cells were lysed with lysis buffer (50mM TrisHCl; 150mM NaCl; 5mM CaCl₂; 0.05% Brij-35 and 1% v/v Triton-X 100). Cells were collected into microfuge tubes and spun at 15 000 rpm for 10 mins. After protein assay to ensure even loading, lysates were incubated with Tris-SDS- β -mercaptoethanol sample buffer and separated on 4-15% Tris-glycine precast electrophoresis gels which were then transferred to PVDF membranes. Membranes were blocked in 5% (w/v) non-fat milk solution with Tris-buffered saline containing 0.1% Tween-20. Primary antibodies were incubated in blocking buffer overnight at 4°C. Antibodies used include: anti-VEGF (1:250; Santa Cruz); and anti-actin (1:2000; Sigma). Once membranes were washed, appropriate secondary antibodies (1:10 000) were also incubated in blocking buffer, and membranes were washed one final time before developing with an enhanced chemiluminescent Western blotting system.

Statistical Analysis

All values are presented as mean $\pm$ SEM, and statistical significance for all sets of data of were calculated using one-way ANOVA followed by Newman-Keuls post-hoc analysis or a Student's t-test where appropriate.

Results:

Characterization of Endothelial Cells

Immunocytochemistry using antibodies recognizing RECA, eNOS and GFAP, demonstrated that cells grown under the conditions outlined, were in fact, endothelial

cells. Approximately 5% of cells obtained from this culture were astrocyte in origin.

Figure 4.2 demonstrates representative photomicrographs of the endothelial cells obtained in these culture conditions.

Figure 4.2. Endothelial Cell Characterization. A. Representative Western blot for GFAP and actin. Lanes loaded with lysate obtained from adult cerebral endothelial cells do not demonstrate any GFAP expression in contrast to lanes loaded with mixed glial culture, containing both astrocytes and microglia, and neonatal astrocytes alone. B. Representative photomicrograph of immunostaining for eNOS, an endothelial cell marker. Inset represents a negative control. C. Representative photomicrograph of immunostaining for RECA-1 a rat specific endothelial cell marker.

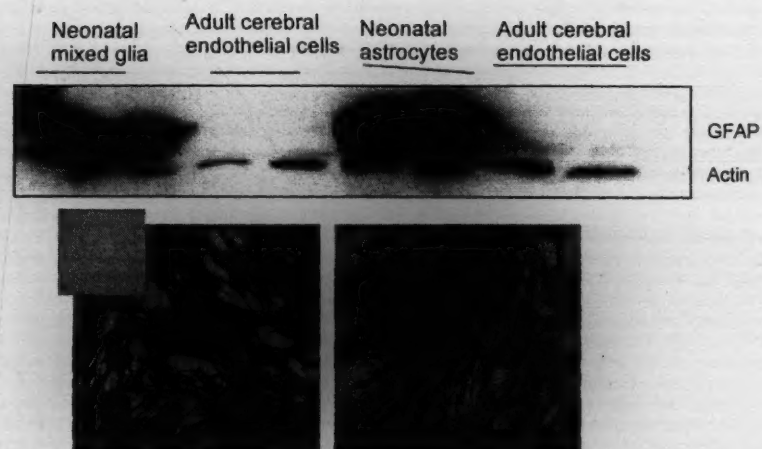
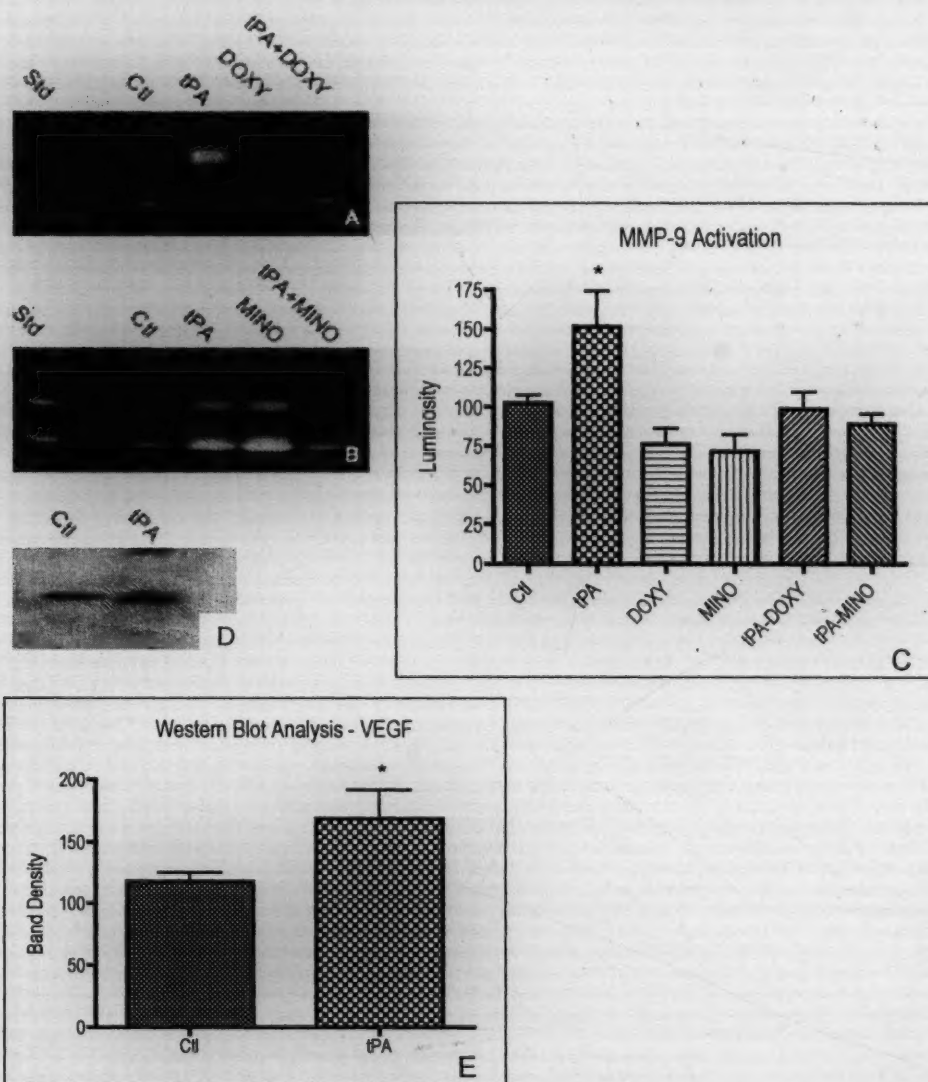


Figure 4.3. A. Representative zymograph from conditioned media from endothelial cell culture treated with tPA and/or DOXY. B. Representative zymograph from similar experiment with conditioned media from cells treated with tPA and/or MINO. C. Histogram depicts the luminosity of gelatin zymography experiment comparing MMP-9 activity of tPA-treated endothelial cells, with DOXY-treated, MINO-treated, tPA + DOXY-treated and tPA + MINO-treated endothelial cells. tPA alone increases gelatinolytic activity while co-incubation with broad-spectrum MMP inhibitors, DOXY and MINO, there is a slight reduction in MMP-9 activation. D. Representative Western Blot analysis of VEGF expression. tPA significantly increases the mobilization of VEGF in compared to wells treated with vehicle, in this case, PBS. E. Histogram depicting the increase in VEGF mobilization.



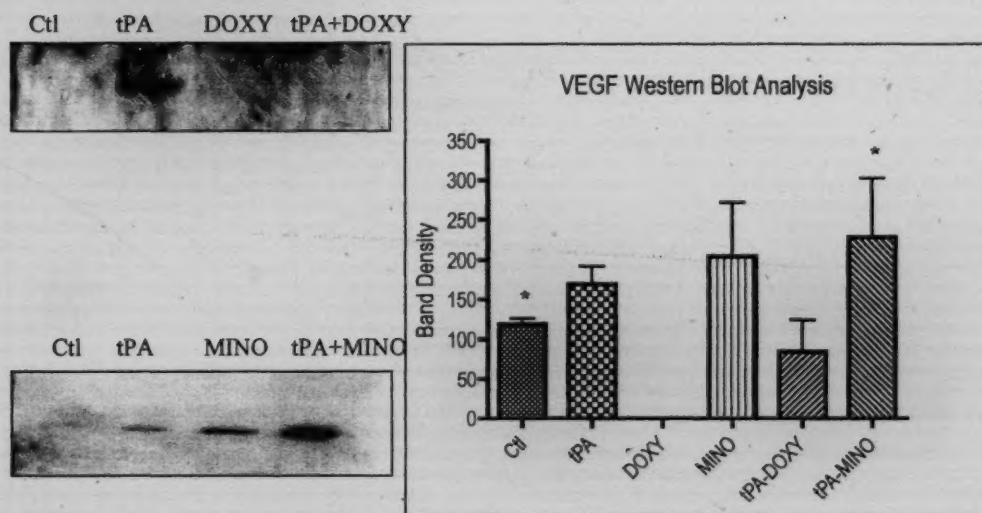
MMP and VEGF expression changes with tPA application

Our *in vivo* data shows that MMP-9 activation is increased in response to tPA administration (please refer to Chapter 3). Here, conditioned media of our RCECs demonstrates the same, tPA-dependent MMP-9 activation (Control: 102.7 ± 4.83 ; tPA: 151.4 ± 22.77 ; numbers represent luminosity of bands $\pm$ SEM). Figure 4.3A-B depicts a representative zymograph demonstrating the tPA-dependent increase in MMP-9 activation, and 4.3C depicts the histogram plotting this data. This increase in MMP expression corresponds to an increase in the mobilization of VEGF in cell lysates compared to vehicle-treated cells (Control: 118.5 ± 7.14 ; tPA: 168.8 ± 23 ; numbers represent band density on Western blot). Figure 4.3E is representative data from Western blotting analysis of VEGF expression in our RCEC lysate.

MMP Inhibition by DOXY Reduces VEGF mobilization

DOXY treatment alone was able to inhibit MMP-9 and subsequently reduce VEGF mobilization as measured by Western blot analysis and ELISA (DOXY: 0.0 ± 0.0). To confirm that DOXY alone was responsible for this inhibition of the mobilization of VEGF, MINO was directly compared to the inhibitory actions of DOXY (DOXY-treated: 0 ± 0 ; MINO-treated: 204.3 ± 66.72 ; numbers represent WB band density). Application of MINO to cultured cells inhibited the activation of MMP-9 to levels similar to DOXY treatment alone (MINO: 71.33 ± 10.9 ; DOXY: 76.22 ± 10.14 ; see Figure 4.4).

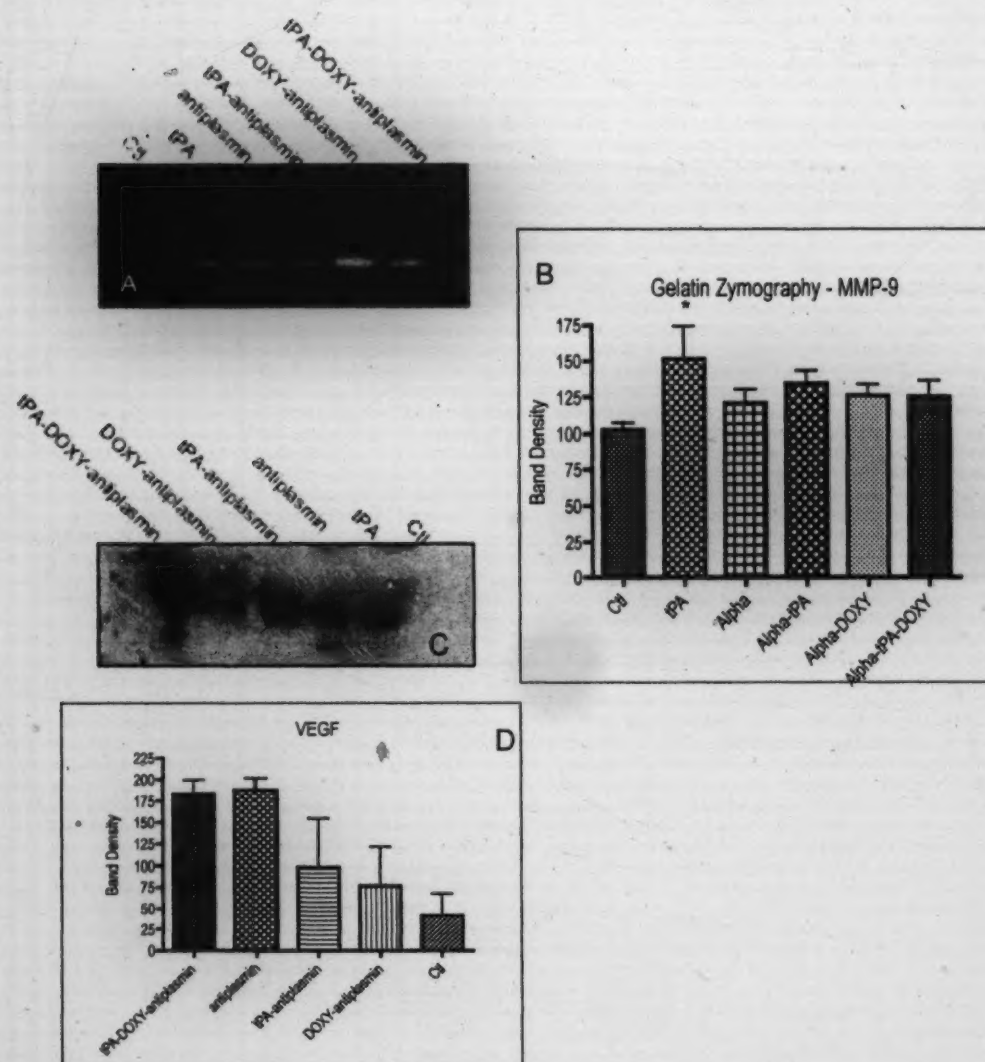
Figure 4.4. MMP and VEGF inhibition by DOXY. A. Representative Western Blot depicting the mobilization of VEGF induced by tPA and the subsequent inhibition by DOXY treatment. B. Representative Western Blot depicting the mobilization of VEGF induced by tPA and the inability of MINO to inhibit this inhibition. C. Histogram depicting data obtained from Western Blot analysis of VEGF mobilization. D. Histogram depicting inhibition of MMP-9 activity (from Figure 4.3) by either DOXY or tPA combined with DOXY results in the reduction of VEGF mobilization. Whereas MMP-9 inhibition with MINO or MINO in combination with tPA, is not effective at inhibiting the mobilization of VEGF.



VEGF mobilization by MMP-9 is plasmin-independent

By adding α_2 -antiplasmin, the plasmin-dependent MMP-9 activation was eliminated (please refer to Figure 4.1). With α_2 -antiplasmin added to culture media, activation of MMP-9 observed in tPA-treated wells, remained unchanged (tPA: 151.4 ± 22.77 ; α_2 -antiplasmin: 120.7 ± 9.75 ; tPA + α_2 -antiplasmin: 134.3 ± 9.0). Following the activation of MMP-9, the release of VEGF also remained unchanged (Control: 118.5 ± 7.14 ; tPA: 168.8 ± 23 ; α_2 -antiplasmin: 188 ± 13.07 ; tPA+ α_2 -antiplasmin: 98 ± 56.63). Figure 4.5C-D display the Western blot data as well as a histogram demonstrating that the density of VEGF expression was not changed among these groups. Thus, the tPA-mediated increase in MMP-9 activation is plasmin-independent.

Figure 4.5. A. Representative zymograph of tPA, DOXY and α_2 -antiplasmin experiment. B. Histogram of gelatin zymography comparing tPA-mediated activation of MMP-9 and α_2 -antiplasmin for the inhibition of plasmin. C. Representative Western blot of VEGF expression from same treatment conditions. D. Histogram of Western blot analysis for VEGF mobilization under the same conditions. This demonstrates that despite plasmin inhibition with an endogenous inhibitor, MMP-9 activity remains as does VEGF mobilization. This indicates a potential compensatory mechanism for the activation of MMP-9.



Discussion:

The experiments herein were designed to examine the molecular mechanisms by which tPA and DOXY mobilize VEGF from the extracellular matrix, in an adult rat brain endothelial cell culture. Figure 4.1 depicts the pathway proposed to be responsible for the observed increase in blood vessels observed on the superolateral surface of DOXY-treated brains in an *in vivo* study described in detail in Chapter 3. It has been established that tPA cleaves the zymogen plasminogen to the active enzyme, plasmin (Pepper, 2001). Plasmin, a serine protease, has been shown to activate MMPs from their pro-form to an active state (Lo et al., 2002; Pfefferkorn and Rosenberg, 2003; Kaur et al., 2004; Lo et al., 2004a). Plasmin itself is able to degrade the extracellular matrix. This degradation allows VEGF to be mobilized, allowing it to bind to one of its two receptors (VEGFR1 and VEGFR2), thereby chemotactically signaling endothelial cells to proliferate and migrate (Pepper, 1997; Zhang et al., 2000; Pepper, 2001; Kaya et al., 2005). Increased activation of MMPs undoubtedly degrades extracellular matrix proteins, again allowing mobilization of VEGF (Pepper, 1997; Zhang et al., 2000; Pepper, 2001). There has been some speculation in the literature of a plasmin-independent ability of exogenous tPA to activate MMPs (Yepes et al., 2003; Benchenane et al., 2005b; Benchenane et al., 2005a). This may occur through the low-density lipoprotein receptor (LRP), which has recently been shown to internalize tPA, once tPA is bound to the receptor (Yepes et al., 2003; Benchenane et al., 2005b; Benchenane et al., 2005a).

In Figure 4.1, the large X on each of the arrows in this pathway represents a point of manipulation of this pathway. Using inhibitors at each step of this cascade, the data demonstrate that tPA alone increases MMP-9 activation and VEGF mobilization while DOXY alone reduces VEGF stimulation. When DOXY is combined with tPA, there is a mobilization of VEGF through a plasmin-independent mechanism. I used a specific plasmin inhibitor to demonstrate that the breakdown of the extracellular matrix allowing VEGF to be free occurs through a plasmin-independent mechanism. To speculate the *in vivo* situation, the half-life of tPA is between 5 and 8 minutes, but it does act to cleave plasminogen into the active plasmin. With this in mind, MMP inhibition would be best achieved between 3 and 6 hours after the administration of tPA. This is in relation to the time-course study examining the acute temporal profile of MMP activation following thrombolysis with tPA, outlined in Chapter 2 (Kelly et al., 2006), which outlined the early destruction of the BBB was induced by MMP-9 activation. While this study demonstrates that the combination of tPA and DOXY is destructive, furthering this breakdown of the BBB, however, it must always be kept in mind that an increase in permeability of the BBB would be necessary for the formation of new blood vessels as they penetrate an otherwise ischemic area.

While MMPs, and specifically the gelatinases, have been largely targeted for their matrix degradation capabilities, this action may not always be deleterious. In the early hours to days following focal cerebral ischemia and particularly following tPA administration, the increased gelatinolytic activity of MMP-9 is largely associated with BBB breakdown (Kelly et al., 2006). This result is further

strengthened in studies which have used synthetically derived MMP inhibitors to successfully reduce the BBB breakdown and decrease infarct size (Romanic et al., 1998; Yrjanheikki et al., 1999; Yao et al., 2004), though none of these studies have combined MMP inhibition with tPA thrombolysis. Just as in the case of neuroblast migration to damaged brain, MMP activation is necessary for angiogenesis to breakdown the extracellular matrix for the migration of proliferating cells (Cornelius et al., 1998; Lamoreaux et al., 1998; Haas and Madri, 1999; Jin et al., 2000). Ideally, there needs to be a balance between reducing MMP activation for rescue of the BBB following ischemia and tPA administration, and enabling regenerative processes which benefit from increased MMP activity.

While it has been shown *in vivo* that the administration of tPA increases MMP expression and activation, it has been proposed that this may occur through a plasmin-independent mechanism (Yepes et al., 2003; Benchenane et al., 2005b; Benchenane et al., 2005a). This mechanism was confirmed by demonstrating the continued MMP-9 activation in cultures treated with α_2 -antiplasmin – a plasmin inhibitor – and further confirmed this by incubating cells with both tPA and α_2 -antiplasmin. Gelatin zymography of conditioned media demonstrated an increase of MMP-9 activation in wells treated with tPA alone and with tPA + α_2 -antiplasmin. This novel study of the interaction between tPA and DOXY, acting in a plasmin-independent manner to increase the availability of VEGF to bind to its receptors represents a preliminary examination at the potential to encourage the proliferation of new blood vessels to the ischemic penumbra.

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Chapter 5:

**Thrombolysis with tPA in an aged model of focal cerebral
ischemia**

Introduction:

The blood-brain barrier (BBB) protects the interface between circulating blood and brain tissue. The BBB is composed of tight junction proteins between capillary endothelial cells and the integrity of the BBB is further strengthened by perivascular astrocytic endfeet and an extracellular matrix (Shah and Mooradian, 1997). Though the BBB is fully developed at birth, it is a dynamic entity which undergoes structural changes throughout development and aging (Shah and Mooradian, 1997; Smith et al., 2006). As we age, these changes include, a reduced number of endothelial cells, increased basement membrane thickness, and changes in microglial responses (Shah and Mooradian, 1997; Campbell et al., 2007).

Advanced age is the single most important risk factor for the development of cardiovascular and cerebrovascular disease (Lakatta and Levy, 2003; Smith et al., 2006). In Canada, 30% of the 50 000 people who suffer from stroke annually are over the age of 80 (Chen et al., 2005). Thus, age-related changes in the integrity of the BBB are an important consideration in the development of neuroprotective strategies.

Tissue plasminogen activator (tPA) has been widely studied in the treatment for acute ischemic stroke. The Neurological Institute of Neurological Diseases in Stroke (NINDS) study of 1995 demonstrated the clinical benefit of the use of tPA over placebo for stroke patients treated within 3 hours of the onset of symptoms (trial, 1995). Since 1995 several clinical trials have been performed to replicate the benefits of tPA, and to extend the therapeutic window (Hacke et al., 1995; Clark et al., 1999; Furlan et al., 1999; Sylaja et al., 2006). While age is not a contraindication

for thrombolysis treatment, age does increase the risk of stroke occurrence and as mentioned, progressive age does see changes in the structural capacity of the BBB. Cardiovascular research has shown an age-related decline in extracellular matrix integrity related to increased matrix metalloproteinase (MMP) activation (Lindsey et al., 2005). MMPs are a large family of zinc-dependent proteases which have been widely studied in both cardiac and cerebral ischemia (Clark et al., 1997; Loftus et al., 2000; Lo et al., 2002; Lalu et al., 2003). The MMPs are primarily responsible for breakdown of the extracellular matrix which occurs following an ischemic event (Kamada et al., 2007). Plasmin and tPA itself are known to be potent activators of MMPs, both *in vitro* and *in vivo* and this may be a mechanism for the hemorrhagic transformation often associated with the administration of tissue plasminogen activator (tPA).

To date, there have been no animal studies investigating the consequences of thrombolytic treatment with tPA in an aged model of focal cerebral ischemia. With particular focus on BBB breakdown by MMPs, this study highlights the vulnerability of the aging BBB and its susceptibility to MMP activation.

Materials and Methods:

Male Sprague-Dawley rats (older than 1 year; 650-780g) were housed in a humidity and temperature-controlled environment with a 12-hour light/dark cycle. The care and use of animals were in compliance with the guidelines as outlined by the Canadian Council for Animal Care. Rectal temperature was maintained at 37°C with a thermostat-controlled heating pad, during surgical procedures. Animals were randomly assigned to one of the following four experimental groups: Ischemia-tPA, Ischemia-Saline, Sham-tPA, or Sham-Saline. Total N=32. To examine the temporal changes in laminin expression and MMP activation, naïve brains from seven-day old rat pups and adult (250-300 g) were obtained and used for this aspect of the study. This supplementary tissue was not subject to any ischemic procedures or drug treatments.

Western Blot Analysis for BBB Components

Equal amounts of protein (25 µg/lane) were loaded and electrophoresed through a 4-15% Tris-glycine gradient gel and transferred to a PVDF membrane. This membrane was blocked by incubation in 5% (w/v) non-fat milk in tris-buffered saline (TBS) containing 0.1% Tween-20 for at least one hour. Primary antibodies recognizing laminin (Sigma; 1:500), zona-occludens-1 (Santa Cruz; 1:500), and occludin (Santa Cruz; 1:750) were incubated overnight at 4°C in blocking buffer. Membranes were the rinsed and incubated with appropriate horseradish peroxidase-linked secondary antibodies for 30 minutes. Membranes were developed using a

chemiluminescent detection kit. Bands were scanned and quantified by densitometry.

Thromboembolic Ischemia

Surgical procedures for the induction of thromboembolic cerebral ischemia have been previously described in Chapter 2. Twenty-four hours following the surgical procedure, animals were euthanized and brains quickly removed and blocked for analyses of neuronal survival, breakdown of BBB components and activation of MMP-2 and -9.

Fluoro-Jade staining for Infarct Volume Measurement

Fluoro-Jade staining was used to assess the extent of neuronal damage induced by focal cerebral ischemia as TTC staining methods proved to be unreliable in this rat population. We found that the results of TTC staining vastly underestimated the extent of infarct in comparison to the amount of BBB permeability observed with laminin immunohistochemistry and IgG extravasation. Thus, brains were flash-frozen in ice-cold isopentane and kept -80°C until brains were sectioned on a cryostat. Sections (20µm) were dehydrated for 3 minutes in 100% ethanol and 70% ethanol, followed by rinsing in ddH₂O. Sections were oxidized in 0.06% KMnO₄ for 15 minutes then incubated in 0.001% Fluoro-Jade solution in 0.1% acetic acid for 30 minutes. Sections were then rinsed and dried at 37°C, for mounting with DPX. Fluoro-Jade is an anionic dye that specifically stains cells undergoing degeneration (Duckworth et al., 2005). Fluorescent staining represents dying neurons and are only

apparent in the ischemic areas in the ipsilateral, right, hemisphere. Infarct volume was measured in Fluoro-Jade stained brains in the same manner described for NeuN immunostaining described in Chapters 2 and 3. Briefly, photomicrographs of each brain section were digitized and the border zone between the infarcted and non-infarcted tissue was outlined using the lasso tool in Photoshop Elements 2.0. The ischemic lesion was calculated as the difference between the area of the non-ischemic hemisphere and the normal area of the ischemic hemisphere. Volume was calculated by multiplying the ischemic lesion by the thickness of the sections (Tureyen et al., 2004; Shen et al., 2006)

Immunohistochemistry

Immunohistochemistry was performed with antibodies recognizing laminin and IgG for quantification and regionalization of BBB breakdown. Cryosections were fixed in formalin and dehydrated through graded alcohols and cleared in xylene. Sections were blocked for endogenous peroxidase and incubated overnight with primary antibody anti-laminin (Sigma, 1:100) followed by incubation with appropriate secondary antibody (1:200). Following secondary antibody incubation, slides were then incubated for 30 minutes with an avidin-biotin complex and visualized with diaminobenzidine (DAB). Negative controls, sections incubated without primary antibody, were run with each assay.

Quantification of laminin immunostaining was performed by counting intact blood vessels (Vosko et al., 2003). Laminin immunostaining was used as a measure of BBB breakdown in conjunction with IgG extravasation described in the next section. Evidence suggests that using tracers to evaluate BBB damage could

underestimate extent of BBB permeability, particularly in non-perfused areas (Todd and Butterworth, 1999; Vosko et al., 2003).

IgG Extravasation

This technique provides a gross measure of BBB breakdown (Calingasan, 1995; Todd and Butterworth, 1999; Kelly et al., 2006) and was used to corroborate laminin immunohistochemical analysis of BBB breakdown. By incubating slides with species-specific IgG, one can microscopically visualize and quantify the extent of leaky blood vessels as the IgG contained within vessels leaks into the surrounding parenchyma. The density of positive brown labeling was selectively measured in the ipsilateral ischemic hemisphere. Sections immediately following in sequence from sections used in laminin immunostaining procedures were used for IgG extravasation experiments.

Gelatin Zymography

Tissue used for gelatin zymography was divided into ipsilateral ischemic and contralateral non-ischemic hemispheres and further dissected into striatal and cortical regions. Brain samples were homogenized in lysis buffer and subject to gelatin zymography as described in Chapters 2 and 3. Gelatin zymography was performed three separate times for each region of each hemisphere. The results of these experiments are provided as an average luminosity of the bands of the three separate trials.

Statistical Analysis

Quantitative data are expressed throughout as mean $\pm$ standard error of the mean (SEM). Statistical comparisons for infarct size, laminin immunostaining and IgG

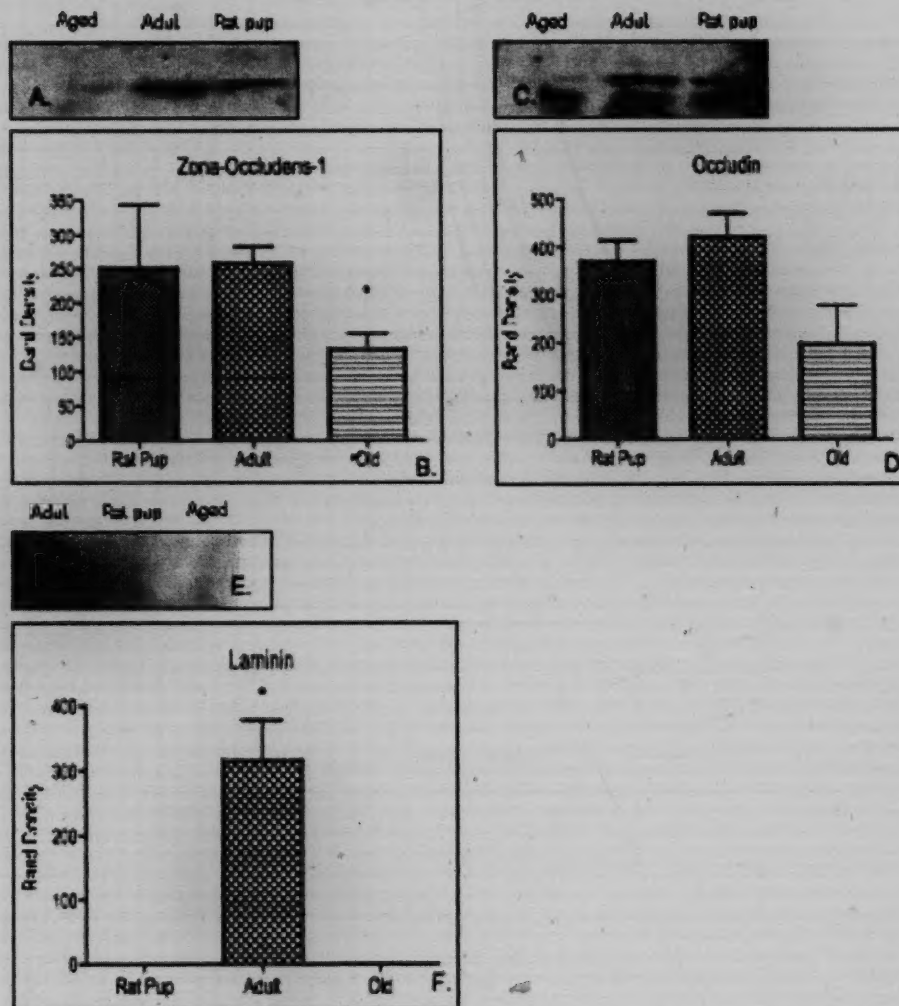
extravasation were performed using Kruskal-Wallis non-parametric analysis followed by Dunn's post-hoc analysis; where appropriate a Mann-Whitney statistical test was employed. This was also used to compare the luminosity of bands for gelatin zymographic analysis. Differences with $p < 0.05$ were considered statistically significant.

Results:

Western Blot for BBB Components

Western blotting data demonstrated an age-dependent decline in the expression of laminin. As shown in Figure 5.1A there was virtually no laminin detected in aged brains despite equal amount of protein loaded. Aged brains demonstrated undetectable levels of laminin in these experiments, whereas the adult tissue and rat pup tissue both demonstrated laminin expression. To corroborate this data we further investigated the expression of two familiar tight junction proteins, zona-occludens-1 and occludin. Here, we see no significant reduction in the expression of occludin was observed, however there was an age-dependent decline in the expression of zona occludens-1 (Figure 5.1 B-D).

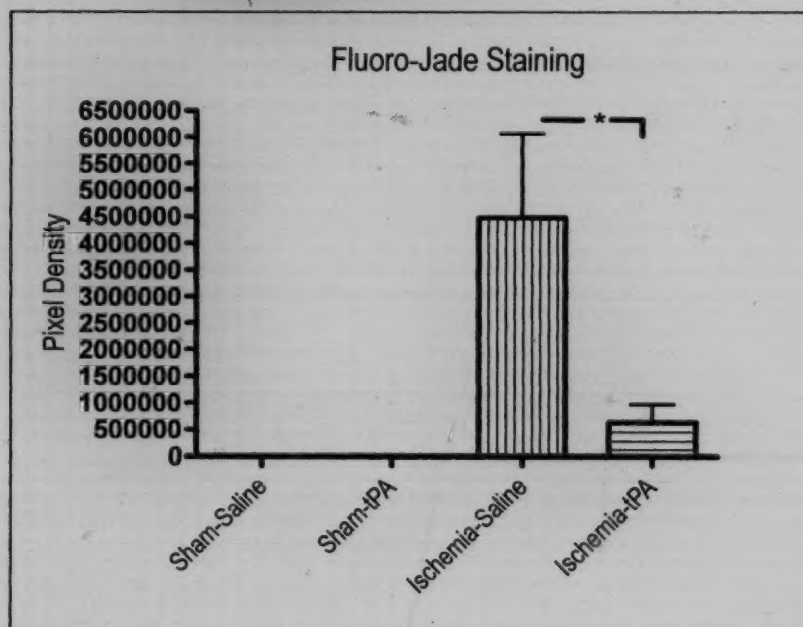
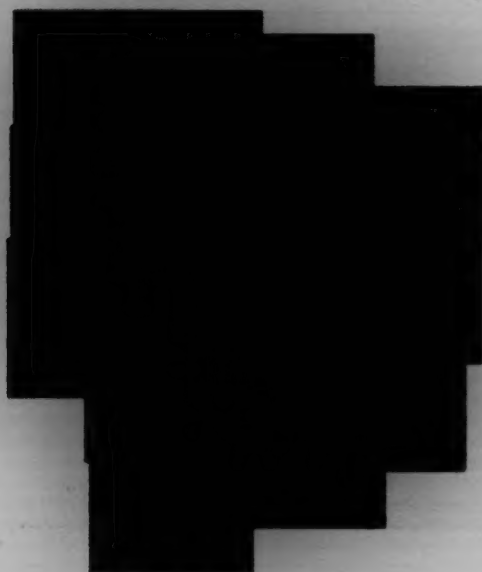
Figure 5.1. Western Blot data. A. Representative blot with an antibody recognizing the tight junction protein zona occluden-1 (220 kDa). Note the lack of ZO-1 expression in the aged brain. B. Histogram of the band density from this data. C. Representative blot with an antibody recognizing occludin (45-84 kDa). Note that there are no significant difference in expression of this tight junction protein throughout the ages studied here. D. Histogram representing the band density of this data. E. Representative blot with an antibody recognizing laminin, an extracellular matrix protein. F. Histogram depicting the difference in laminin expression through the three age points studied here. Only the adult brain has detectable levels of laminin protein.



Fluoro-Jade

In all sham-operated animals, there was no positive Fluoro-Jade staining, indicating that there is no neuronal cell death in either hemisphere of rats aged more than one year whether or not they receive tPA, in an uncompromised brain. In ischemic animals, Fluoro-Jade staining was apparent within the right hemisphere and was localized to the lateral striatum and lateral cortex. These are the regions primarily supplied by the middle cerebral artery and would be expected to be affected by the occlusion of this vessel. A representative photomicrograph of Fluoro-Jade staining observed in ischemic animals can be seen in Figure 5.2A. A histogram of Fluoro-Jade staining through all four groups can be seen in Figure 5.2B. This histogram displays the pixel density of Fluoro-Jade positive areas in ischemic and non-ischemic animals.

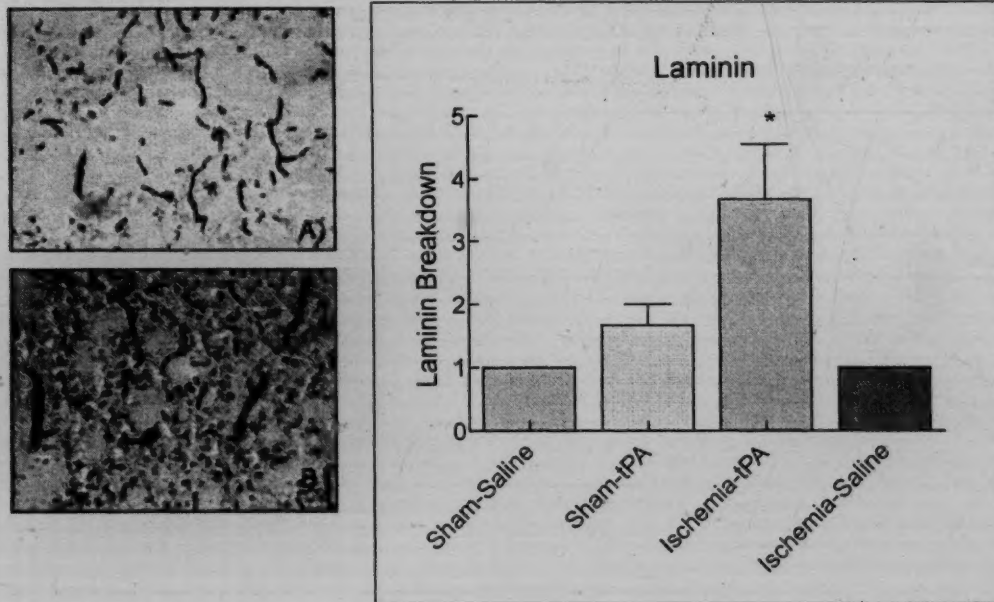
Figure 5.2. Fluoro-Jade quantification of infarct size. A. Representative photomicrograph of Fluoro-Jade stained coronal section. Traced areas demonstrate ischemic tissue. Note the patchy appearance of the infarcted tissue. B. Histogram of Fluoro-Jade measurement of infarct volume.



Laminin immunohistochemistry

Ischemic animals treated with tPA demonstrated the greatest amount of loss of laminin immunostaining throughout the ipsilateral hemisphere (3.67 ± 0.89 ; values represent a measure of the extent of laminin loss). Vehicle-treated ischemic animals were similar to sham-operated animals in the extent of lost laminin throughout the ischemic hemisphere. It was noted that in sham-operated animals, there was an obvious reduction in the amount of positive staining for laminin throughout the sections examined compared to any adult laminin immunostaining performed in our lab (data not shown).

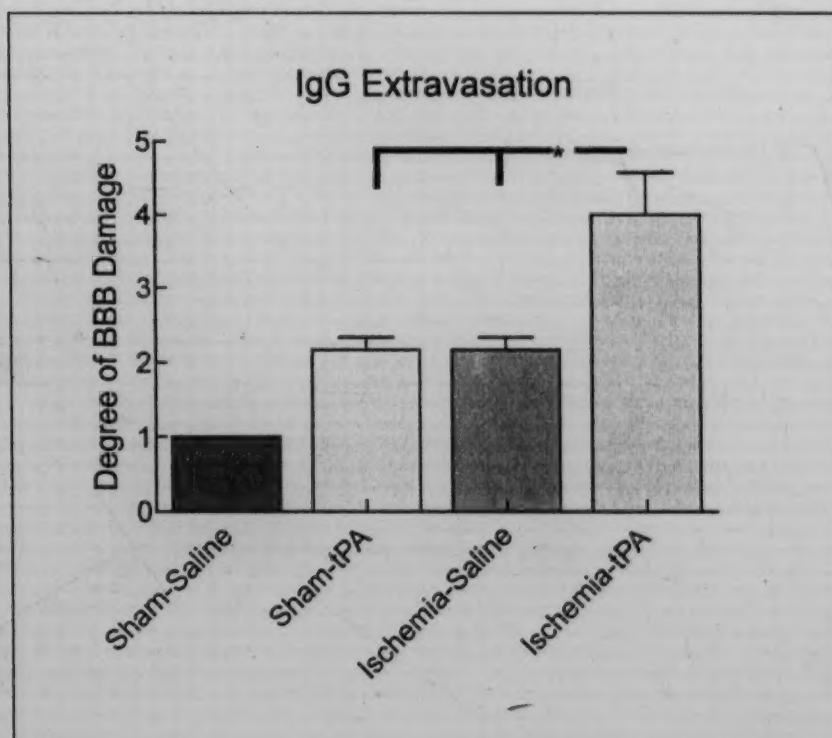
Figure 5.3. Laminin Immunohistochemistry. A. Representative photomicrograph of untreated, sham animal aged more than one year. B. Representative photomicrograph of untreated, sham, adult animal. C. Histogram depicting the increased breakdown of laminin in tPA-treated ischemic animals.



IgG Extravasation

To corroborate laminin immunohistochemistry results, IgG extravasation was used. This experiment demonstrated similar results to laminin immunostaining in that, tPA-treated ischemic animals displayed the greatest amount of IgG leaking into the surrounding brain parenchyma. Again, vehicle-treated ischemic animals were virtually indistinguishable from sham-operated animals in the extent of IgG extravasation observed.

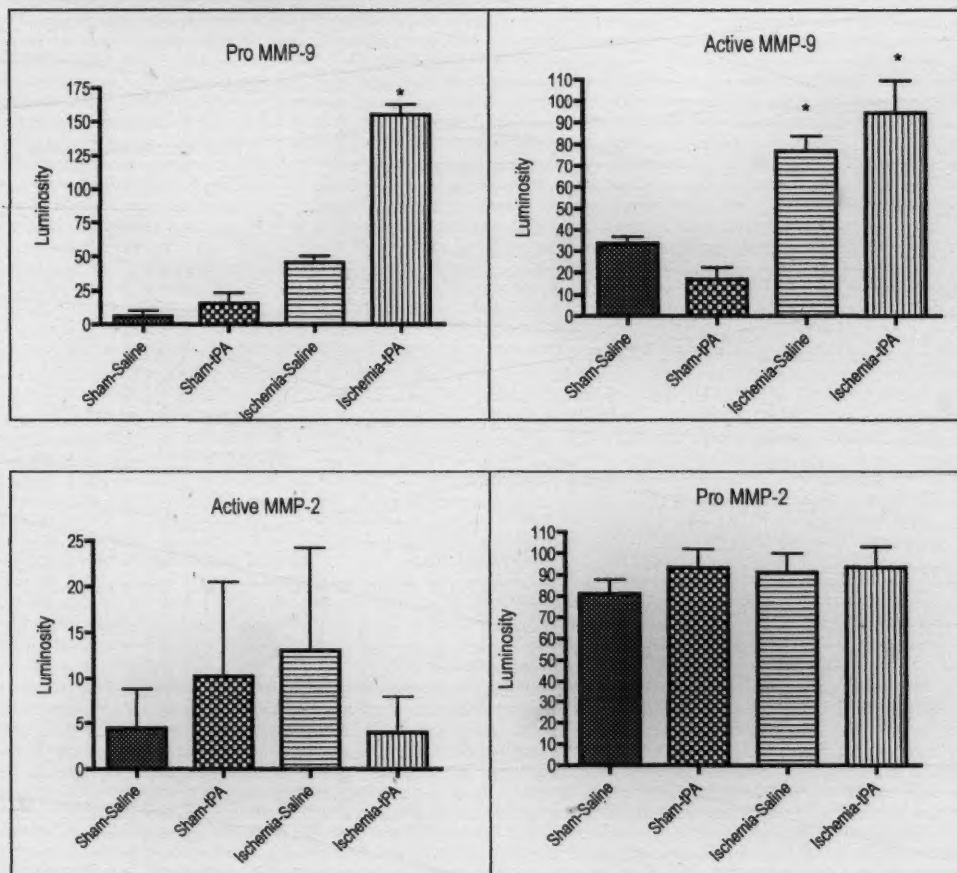
Figure 5.4. Histogram depicting the extent of IgG extravasation throughout the ischemic hemisphere in aged brains. Note that tPA-treated ischemic brains had the most amount of IgG extravasation, representing BBB breakdown.



Gelatin Zymography

The activation of MMP-9 was increased with tPA-treatment in ischemic animals (94.58 ± 15.00 ; 16.9 ± 5.42 ; values represent luminosity of active MMP-9 bands $\pm$ SEM) compared to tPA-treated sham animals, but it was not significantly higher than vehicle-treated ischemic animals (76.96 ± 6.79). This is in contrast to levels of pro-MMP-9 where tPA-treated ischemic animals had the highest amount of pro-MMP-9 than any other group tested (155.1 ± 7.62). In this study of aged animals we found no treatment-dependent changes of MMP-2, in either its pro or active form. This was expected based on the data described in Chapters 2 and 3 of this thesis, which demonstrated the long latency to activation of MMP-2 in comparison to MMP-9. A representative zymograph is displayed in Figure 5.5A, along with a histogram demonstrating the change in MMP-9 levels induced by tPA-treatment following a focal ischemic event. Figure 5.5C represents a zymograph of MMP activation in rat pup, adult rat and aged rat brain. The tissue used to obtain this data, was untreated.

Figure 5.5. Gelatin Zymography. A. Representative zymograph from aging study, comparing the four treatment groups. B. Histogram of this data for MMP-2 and MMP-9 activation and expression. C. Representative zymograph from comparison of MMP expression and activation between rat pup, adult rat and rat aged older than one year.



Discussion:

The use of tPA as a thrombolytic agent has been approved for human use since 1996, though its use remains controversial (trial, 1995). This is largely due to its association with hemorrhagic transformation following thrombolysis in some patients. As the downstream cellular pathways of tPA are elucidated, the side effect profile of tPA can be improved and ultimately be made available to more of the population who suffer from stroke. There has been a lack of focus on the administration of tPA to an older population, such as those older than 80 years of age (Sylaja et al., 2006). This population represents approximately 30% of all strokes and is also the fastest growing demographic in developed countries (Sylaja et al., 2006). One study specifically examined the difference in outcome following tPA thrombolysis for focal cerebral ischemia in both a population above the age of 80 years and a younger population, below 70 years (trial, 1995; Chen et al., 2005). In the NINDS trial, age was not found to be an independent risk factor for symptomatic hemorrhage in tPA-treated patients and this was replicated in the study by Chen et al., in 2005. Here, regardless of age, patients receiving tPA demonstrated similar recanalization and similar recovery rates following intravenous tPA treatment (Chen et al., 2005). In theory, there are several factors which may contribute to the hemorrhagic transformation elderly patients such as a frail vasculature, an impaired rate of tPA clearance or cerebral amyloid angiopathy (Tanne et al., 2006).

This study was designed to examine the effects of tPA on the activation of MMPs and the permeability of the BBB in rats aged older one year. There was a

significant neuroprotective effect of tPA in these aged rats and tPA also drastically increased the permeability of the BBB and was also shown to increase MMP-9 activation. With more time following the focal ischemic insult, the protective effect of tPA would be expected to become less beneficial as the BBB continues to be degraded by the increased activation of MMPs. This was also demonstrated in the earlier presented study with an adult model of focal cerebral ischemia (Chapter 3). MMP-2 has a longer latency to activation and its expression and activation has been shown to be increased following treatment with tPA (Kelly et al., 2007; under peer review). As the evolution of the stroke proceeds and the BBB is exposed to the activation of MMP-2, following the early activation of MMP-9, it is possible that there would be a further increase in BBB permeability thus negating the protective effects of tPA.

Several age-related changes occur in the cerebral microvasculature, including, a reduction in the number of endothelial cells, an increase in the thickness of the basement membrane surrounding cerebral blood vessels, also changes in the lumen diameter of these vessels (Shah and Mooradian, 1997; Campbell et al., 2007). However, it has been shown that there is not an increase in BBB permeability with increased age, as shown by experiments using C¹⁴ sucrose and horseradish peroxidase (Shah and Mooradian, 1997). A major determinant of BBB integrity are the tight junction proteins and increased age has been shown to be associated with a reduction in the level of some, but not all, tight junction proteins (Campbell et al., 2007). Though there is no direct evidence for a compromised BBB with increased age, there is a loss of the structural components of the basement membrane and the

tight junction proteins which provide integrity to the BBB. In this study an age-dependent reduction in zona-occludens-1 but not occludin was observed. Inherently this indicates that there is a selective loss of specific proteins that are integral to the BBB. By providing an exogenous MMP activator in the case of plasmin, this could and as indicated by this study, does, amplify the vulnerability of the aged BBB. Thus, this study fills a gap in the literature regarding the age-related changes in the integral proteins of the BBB and examines the mechanism of tPA-induced BBB breakdown in focal cerebral ischemia. As the cerebral vasculature ages and the tight junction proteins between endothelial cells are lost, there is a change in the vulnerability of the BBB. Although tPA was found here to be neuroprotective, as expected, it drastically changed the permeability of the BBB, while increasing the activation of MMP-9. There was no change in the expression of MMPs as the animals age but with an innately compromised vasculature, the activation of MMPs through the exogenous application of plasmin, the activation of MMPs would be much more devastating in terms of their proteolytic activity. It is hypothesized that inhibition of MMPs in combination with tPA thrombolysis would reduce the dramatic BBB breakdown seen in this already vascularly compromised age group.

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Chapter 6:
General Discussion

Discussion:

The goal of the studies included in this thesis was to examine the mechanisms underlying BBB breakdown following tPA administration for the treatment of acute cerebral ischemia in an animal model. The progression of these studies has allowed for the full understanding of BBB breakdown and the potential conversion to a hemorrhagic event following clot lysis with tPA.

The first study in Chapter 2, examined the time-course of acute MMP activation in the first 24 hours after a focal ischemic insult. The conclusions from this study were that MMP-9 activity was increased 6, and 12 hours after ischemia, and this was further increased with tPA administration. The time course experiments were the first to outline MMP activation and BBB breakdown following tPA thrombolysis throughout the first 24 hours following focal cerebral ischemia.

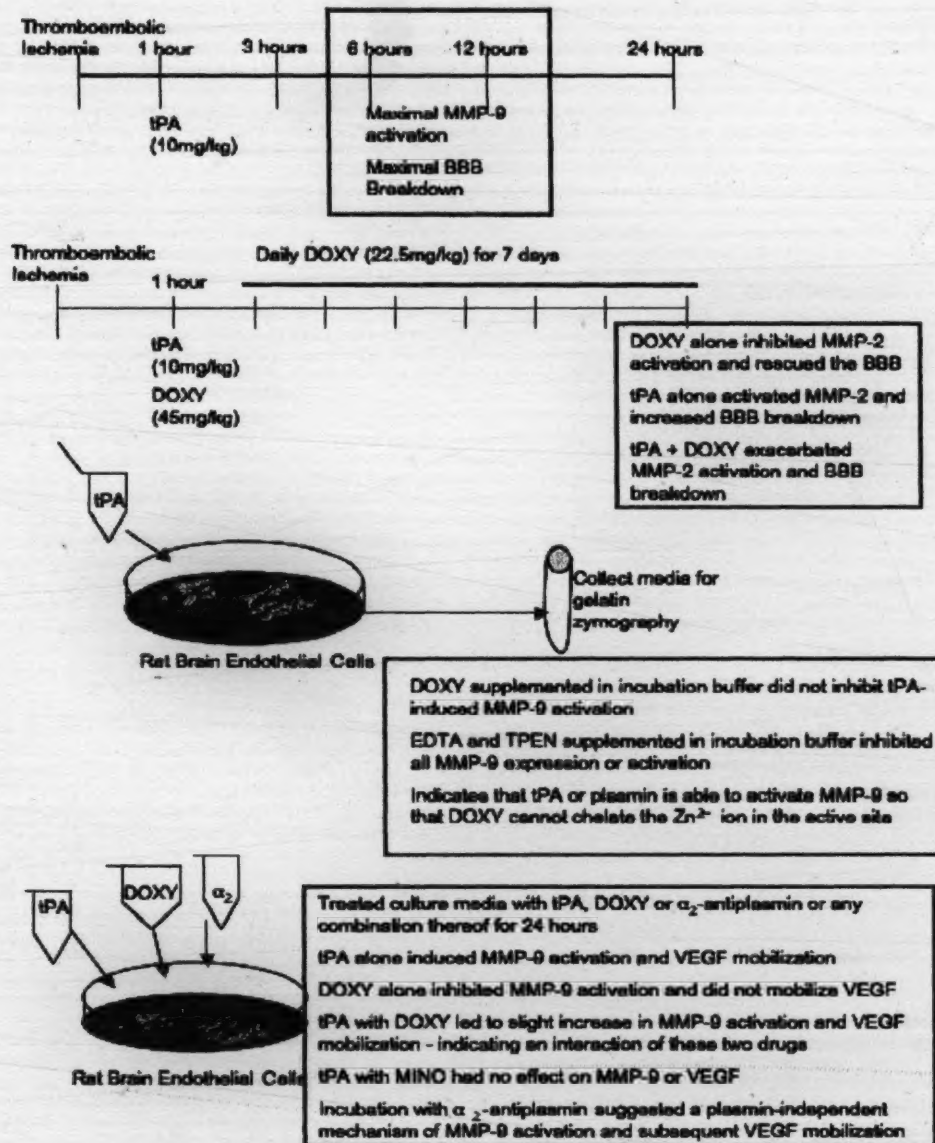
As many specifically directed MMP inhibitors have largely failed in animal experiments, they have been deemed inappropriate for clinical use (Overall and Kleinfeld, 2006). Doxycycline (DOXY) is a readily available, approved compound with an approved safety profile that can be easily administered (Golub et al., 1983). In recent years DOXY and other tetracycline derivatives have been found to possess MMP inhibitory actions in addition to their traditional use as antimicrobial agents (Golub et al., 1983; Golub et al., 1987; Golub et al., 1998; Yrjanheikki et al., 1999; Koistinaho et al., 2005). The combination of tPA thrombolysis and MMP inhibition with DOXY as described in Chapter 3 had never been examined before this study. The focus of this study was the long term effects of tPA and the added effects of MMP inhibition with DOXY. The combined treatment of tPA thrombolysis with

DOXY was hypothesized to be efficient for clot lysis while limiting the degree of BBB breakdown that occurs as a result of increased MMP activity induced by tPA administration. However, MMP inhibition with DOXY for seven days following tPA thrombolysis was found to be detrimental and worsened BBB breakdown, increasing neuronal loss and exacerbating MMP-2 activation.

To further elucidate the tPA-DOXY interaction, conditioned media from endothelial cell cultures was used and MMP activation was examined following incubation with tPA. The purpose of this study was to investigate the hypothesis that tPA can activate MMPs by altering the conformation of the MMP molecule so the propeptide domain remains. This would be expected to hinder the MMP inhibitory properties of DOXY as the active site and the Zn^{2+} ion would be shielded from inhibition (Rajagopalan et al., 1996; Hartung and Kieseier, 2000). To address this hypothesis, gelatin zymography was performed and zymograms were incubated with incubation buffer supplemented with different known chelating agents or MMP inhibitors. Results from these experiments demonstrated that DOXY was unable to inhibit MMP activation induced by tPA. The same result was not obtained when zymogram incubation buffer was supplemented with MINO. Thus further confirming the literature which indicates that DOXY and MINO have differing mechanisms of inhibiting the gelatinases. Results from this study which show that the interaction between tPA and DOXY exacerbates BBB breakdown and enhances MMP activation can be explained by the known increase in conversion of plasminogen to plasmin induced by DOXY. However, this mechanism does not fully explain the surprising increase in angiogenesis on the superolateral of ischemic

brains treated with DOXY (data not shown). DOXY has been shown in several experimental models to inhibit VEGF mobilization thereby reducing the proliferation and migration of endothelial cells, providing a means to limit tumor growth. I would speculate that the biphasic opening of the BBB which occurs following any ischemic event would provide an explanation. This long term study did not examine the acute effects of tPA thrombolysis combined with MMP inhibition by DOXY, the focus was primarily seven days after injury. The longer latency to activation of MMP-2 may represent a mechanism for the formation of new blood vessels within the penumbra. As the MMP-2 activation increases 5-7 days following ischemia, it may serve to breakdown the extracellular matrix, further compromising the BBB, but also allowing VEGF molecules to be mobilized to bind to one of their receptors. This mechanism could be of great therapeutic potential for returning blood flow to areas of ischemic damage that would benefit from increased collateral circulation. To investigate this, cerebral endothelial cells obtained from adult rat brain were cultured and incubated with tPA to induce MMP activation. DOXY or MINO were used to inhibit MMP activation and α_2 -antiplasmin was used to inhibit the enzymatic action of plasmin, in order to determine if the effects on the gelatinases and subsequently on VEGF, were due to tPA or plasmin. Figure 6.1 depicts the experiments as a schematic.

Figure 6.1. Schematic to summarize relationship between first three studies



The *in vivo* results from the aforementioned studies have established that tPA is able to increase the activation of MMP-2 and -9. This increased MMP activity has long been attributed to plasmin, and while it is true that plasmin is responsible for clot lysis, it could be that tPA itself could be activating MMPs. The second study included in this thesis proposes that tPA could activate MMP-2 in such a way that renders DOXY unable to exert its inhibitory effects. For the third study I sought to not only determine how tPA and DOXY interact to mobilize VEGF but also to answer the question of which molecule is responsible for this activation, tPA or plasmin? The use of α_2 -antiplasmin eliminated the possibility that plasmin would be responsible for observed MMP activation.

As outlined in Chapter 4, tPA alone increases MMP-9 activation leading to increased VEGF mobilization. DOXY alone inhibited the mobilization of VEGF from the extracellular matrix, and inhibited MMP-9 activation. The combined effect of tPA with DOXY slightly increased VEGF mobilization. Studies performed with MINO alone and in combination with tPA demonstrated no changes in VEGF mobilization from control samples. These results further strengthen the hypothesis that DOXY and MINO have different downstream effectors. The mobilization of VEGF induced by tPA is indeed a plasmin-independent mechanism as the co-incubation with a plasmin inhibitor, α_2 -antiplasmin abolished the heightened VEGF observed in tPA-treated cells.

This thesis provides a detailed study of the mechanism of BBB breakdown caused by tPA-induced MMP activity. Each chapter provides a scaffold for the subsequent section and it is concluded with a study examining these same parameters

in an aged model of cerebral ischemia. It is important to keep in mind that significant vascular changes occur within the cerebral vasculature with increased age. The administration of the thrombolytic agent, tPA, has been shown here to further degrade the BBB than what was observed in the adult animals described in previous chapters, due to an expected increase in MMP-9 activity. In the strict examination of infarct volume, although tPA may be neuroprotective, this potentially harmful compound induces profound changes to downstream enzymatic pathways and the BBB.

Future Directions

Although tPA has been extensively studied in neuroprotective paradigms for focal cerebral ischemia, it has never been shown to differentially activate MMPs (Graham, 2003; Lo et al., 2004a; Kahles et al., 2005; Liot et al., 2006). Altered MMP conformation, allowing for the pro-form of the enzyme to be proteolytically active has been demonstrated in the presence of NO donors and thiolating agents, but has not been attributed to any other compound (Gu et al., 2002; Lalu et al., 2005). The results within this thesis provide a mechanism for the observed deleterious effects of tPA and DOXY on the ischemic brain. It also enables an explanation for the observed increased in vascularization of the ischemic hemisphere in DOXY-treated animals. Manipulation of this interaction to find the ideal balance between clot lysis, BBB rescue and increasing angiogenesis in the ischemic hemisphere is a pathway that has not yet been explored. By providing a means to enhance cerebral

blood flow to the penumbral area following clot lysis with tPA, it may be that an increased number of stroke patients would not only receive thrombolytic therapy but also would not be at risk of hemorrhagic transformation. All of these parameters also need to be examined in an aged model of ischemia, as this is the prevalent stroke population. With an age-related decline in tight junction protein expression and a loss of structural proteins, the combination of tPA and DOXY to an aged ischemic brain would be expected to be extremely harmful. However, signaling the induction of angiogenesis may prove to be more efficient in an aged brain, due to the weakened barrier that exists surrounding blood vessels. The mobilization of VEGF is expected to be heightened and this may be another aspect of therapeutic intervention to be explored.

My thesis has focused on the activation of MMPs following tPA administration, and the dynamics of the BBB after stroke. The finding that tPA, and likely not the active enzyme plasmin, is able to alter the conformation of the gelatinases to render them active while remaining in a pro-form is intriguing in that this has only been previously shown to occur in an oxidative stress paradigm. That other compounds are able to induce this conformational change in MMPs is novel, added to this is the combined effect of DOXY in this setting. The tPA-DOXY interaction which proved to be detrimental to the BBB, and the result that DOXY alone is unable to inhibit MMPs which have not been cleaved of their pro-peptide region is also novel. The results herein provide a starting point for the further investigation of BBB rescue following tPA administration and the link that this has to angiogenesis and the formation of collateral circulation to an ischemic region.

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